MORPHOLOGICAL AND EXPERIMENTAL STUDIES ON KERATINISATION

Thesis submitted for the degree of Doctor of Medicine of the University of Glasgow

By

PETER PULLAR, M.B., Ch.B.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

INTRODUCTION

This thesis, divided into five parts describes abnormal keratinisation in human skin and keratin formation under various experimental conditions.

Part I is concerned with the normal process of keratin formation in human skin. Apart from its intrinsic interest, this section is important since it compares abnormal and experimental processes which are described later. The chemical and physical properties of keratin relevant to the present study are briefly summarised and an account is given of the histochemistry of the normal process. The histochemical methods used are evaluated and their relevance to the present study discussed.

Part II deals with abnormal keratinisation in human skin and my interest in this subject stems from this work. Hyperkeratosis and dyskeratosis are the two main types of abnormality found in human skin lesions. These processes are illustrated by an account of the morphology and histochemistry of infective warts.

The remainder of the thesis consists of three separate but related groups of experimental work. Part III describes experimental studies with the material discussed in Part II.

Bacteriologically sterile cell free filtrates of distinct types of skin papillomata were inoculated on the chorio-allantoic membrane of the developing egg in an attempt to produce distinctive lesions. Although this aim was not achieved, the membrane reacted

to the various inocula by squamous metaplasia with the formation of keratin. The morphology and histochemistry of the process is described. Human skin was also grafted on to the membrane and its behaviour and reaction to inocula with particular reference to keratin formation are also described.

In Part IV the periodic keratinisation of the vaginal epithelium in mice during the oestrus cycle is considered. This study results from the observation that the process is abolished in mice which have been grafted with a transplantable corticotrophin secreting tumour of pituitary origin. The morphology and histochemistry of vaginal keratinisation in normal LAF 1 mice is described and compared with the process in mice bearing corticotrophin and thyrotrophin secreting tumours. The effects of adrenalectomy and gonadectomy on vaginal keratinisation in such mice is considered in an attempt to elucidate the hormonal mechanisms involved. The effects of steroid hormones on vaginal epithelium in mice is discussed. This leads to a consideration of their effects on epithelium from various sources and to the subject matter of Part V.

Part V is the largest section and is concerned with the process of keratin formation <u>in vitro</u>. The techniques of cell and organ culture have been utilised. The tissues cultured include human adult and foetal skin, foetal mouse skin, infantile and adult mouse

vaginal epithelium. These tissues are shown to form keratin *in vitro* and the morphological and histochemical findings at various stages in the process are described. In the previous sections some consideration has been given to those factors which are thought to influence the process of keratin formation *in vivo*. This particularly concerns Part IV where the interactions of the various steroids and their effects on vaginal keratinization have been discussed. The effect of incorporating these and related substances in the culture medium is described together with an account of the effect of non-steroids, notably Vitamin A which are known to affect keratin formation *in vivo*.

To enhance the continuity of the text, tabulated data, technical methods not described in the text and illustrations are given in a separate volume.

TABLE OF CONTENTS

PART I

THE FORMATION OF KERATIN IN NORMAL HUMAN SKIN

THE CHEMICAL COMPOSITION OF KERATIN

The term keratin refers to a group of tissue proteins of ectodermal origin which occur widely in vertebrates. Compared with other proteins, relatively little is known about their detailed structure, composition or formation. They are resistant to digestion by pepsin and trypsin and are insoluble in dilute acids, alkalies and organic solvents. This lack of solubility renders them less amenable to study than other proteins.

Block (1951) has shown that the total proteins of a homologous group have a remarkably constant amino-acid composition. In the case of keratins, however, this generalisation does not apply. Thus in Table I (p. 175) (Ward and Lundgren, 1954) it is seen that the amino-acid composition of keratins from various species differs. Further, a variation in composition also exists in keratin from different sites in the same species, e.g., human skin and hair.

Flesch (1958) has emphasised that caution is indicated when interpreting data in this field. In addition to the inherent error of the methods used, which he estimates at 5-10%, he points out that abnormal material is often substituted for epidermal keratin. Thus, material referred to as epithelium by Block (1951) consisted of scrapings from a case of exfoliative dermatitis.

It has also been pointed out by Rothman (1954) that the term

keratin often refers to material which has been obtained from keratinous structures (hair, nails and horny layer of skin) by more or less thorough purification and it is therefore regretable that the terms keratin and keratinous structures have been used as if they were synonymous.

The work of Muting et al. (1955) escapes this stricture. They compared the amino acid composition of human epidermal keratin (from callus) with that of heat separated epidermis. Their findings are summarised in Table II (p. 176). Only two changes are established by this data as occurring during epidermal keratinisation - the disappearance of hydroxyproline and a variable and inconstant rise in cystine.

Despite the various objections which have been raised, one must conclude from a consideration of the relevant literature that there is probably only one constant chemical feature which characterises the hydrolytic products of keratins compared with other proteins. This is a high content of cystine.

THE PHYSICAL PROPERTIES OF KERATIN

An immense volume of literature has emerged on this topic, particularly in regard to wool fibres. The basic structure of keratins has been elucidated by Astbury by the use of the X-ray diffraction technique (1933a and b; Astbury and Woods, 1934). Hair keratin has been shown by this technique to exist in two forms of stereo-isomer 'α', the normal folded type and 'β' which is extended.

Giroud and Champetier (1936) found that both the keratinised and Malpighian layers of the horse burr exhibited a periodicity identical to the α pattern of the unstretched human hair. Mercer (1949) found that in hair the α pattern appeared in the upper Malpighian layer and persisted unchanged throughout the 'keratogenous' zone and into the hair itself. Giroud and Leblond (1951) pointed out that this pattern is not present in epithelial organs which do not contain 'tonofibrils' and suggested that these structures are the support for the characteristic protein with an α pattern. This protein is a keratin precursor which becomes fully keratinised in the region of passage from the Malpighian layer without change in the fibrous skeleton, at least as far as the X-ray periodicity is concerned.

Thus in the normal state most keratins yield an α spectogram pattern. They probably consist of parallel polypeptide chains

which are folded in a plane transverse to the side chains which link them (Rothman, 1954). These side chains are of considerable importance in the process of keratinisation. Astbury (1942), for instance, states that 50% of the weight of wool is represented by the side chains. The three main types of cross-linkages between neighbouring polypeptide chains are shown in Figure 1 (p. 200).

Briefly they are:- 1. Salt cross linkages. 2. Hydrogen bonds and bridges. 3. Disulphide bonds.

Extensive investigations have been carried out into the importance of these linkages, notably by Speakman and his colleagues (Speakman, 1936; Speakman and Townend, 1937; Speakman and Stott, 1938). They attribute many of the physical properties of wool keratin to the disulphide bond. Other investigators have suggested a more important role for hydrogen bonding. Amongst these is Alexander (1951) who has conducted numerous contraction experiments with hydrogen and disulphide bond splitting agents. He, however, also attributes an essential role in keratinisation to the disulphide groups as he postulates the wool protein molecules leave the hair root in the form of a gel which contains free sulphydryl groups. The micelles become cross-linked by the formation of disulphide bonds, whereupon the material becomes a keratinised fibre.

THE PROCESS OF KERATIN FORMATION

A. INTRODUCTION

The conversion of living cells into an acellular mass of apparently homogeneous material - keratin - is known as the process of keratinisation or cornification. Little is know for certain about this process. To this lack of basic information there must be added the difficulty in correlating the morphological changes with what is known about the chemical alterations involved.

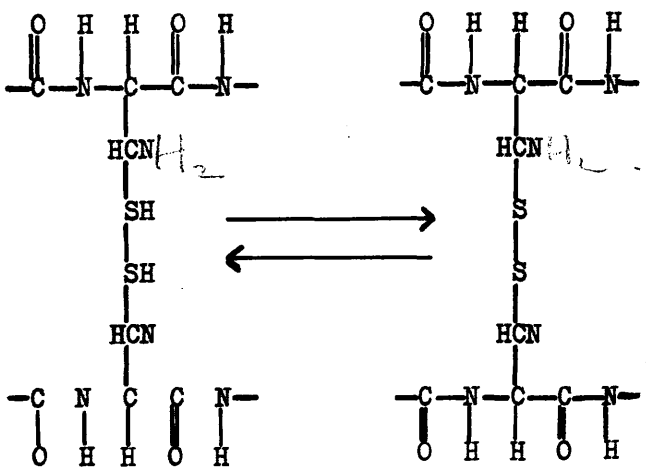
In the remainder of this section a résumé will be given of what is known about the chemical processes involved. From the preceding brief consideration of the chemical and physical properties of keratin, it emerges that the role of sulphur containing amino-acids is of paramount importance. The various histochemical methods available for the demonstration of sulphydryl and disulphide groups of these amino-acids will be considered in some detail and their specificity evaluated. The distribution of these groups in normal human skin is also considered in some detail.

The rationale of the histochemical demonstration and the distribution of other substances is also considered briefly. Technical details are given in the appendix (p.289).

B. THE CHEMISTRY OF THE TRANSFORMATION OF EPIDERMAL PROTEINS INTO KERATIN.

As mentioned earlier, from a comparison of the chemical analysis of the amino-acid composition of keratins and their precursors (the cytoplasmic proteins of the epithelial cells), it emerges that the only constant chemical difference is the higher cystine content of the keratins. This increase in cystine content is associated with a decrease in sulphydryl containing amino-acids. Again, the largest part of the cystine sulphur in the keratin molecule is present in the form of disulphide cross-linkages. Rothman (1954) summarises the position when he states that there can be little doubt that during keratinisation sulphydryl containing amino-acid residues facing each other on neighbouring polypeptide chains will close to form a disulphide cross-linkage.

The formation of such a disulphide bond is illustrated by Cohn & Edsall (1943)



In this figure the SH containing amino-acid residues represent cysteine and when their adjacent SH groups are oxidised to form a S-S group, they will form a cystine residue which bridges over two polypeptide chains.

As Rothman (1954) points out, it is difficult to account for the lessening of the amount of methionine as its sulphur is not labile. A possible explanation may be its utilisation also for the formation of disulphide cross-linkages. The main steps may be as described by Marston (1946) and include the demethylation of methionine to homocysteine and the formation of an addition product with serine. Finally there is incorporation of its sulphur into the SS cross-linkages.

C. MORPHOLOGICAL ASPECTS OF KERATINISATION

The histological appearances are well known and yield little information as to the basic processes involved. It is important, as Rothman (1954) points out, to recall that the process includes not only the conversion of cell proteins into keratin fibres but also a complete disintegration of the keratinising cell. Under normal circumstances, in a tissue such as skin, the two processes proceed simultaneously and by the time the Stratum Corneum is reached there is apparently complete decomposition of both cytoplasm and nucleus. The transition from the squamous (malpighian) cell layer to the acellular refractile keratin layer is abrupt, the

zones being separated generally by one or at the most two layers of cells of the Stratum Granulosum (figure 2, p. 201).

This may be regarded as a transitional zone. The cells are filled with the so-called keratohyalin which, as is well established, differs in staining characteristics from both keratin and hyalin. From numerous and often conflicting reports (e.g., Smith & Parkhurst, 1949; Lansing & Opdyke, 1950; Leuchtenberger & Lund, 1951), their precise chemical nature remains in doubt. Indeed little evidence remains that they are keratin precursors as was formerly supposed and they probably are cellular degeneration products. The tonofibrils which Unna & Schumacher (1925) described under the heading of spongioplasma and which have been implicated frequently in the process of keratin formation are also structures about which little definite is known. Rothman (1954) summarises the position by saying - "their morphology, relation to intercellular spines, continuity beyond cell boundaries and even very existence are not established on morphological grounds, in spite of the fabulous development of morphological methods such as phase or electron microscopy".

D. HISTOCHEMISTRY OF KERATINISATION.

1. Introduction. This section deals with the histochemical findings in normal human adult skin.

As discussed in the previous sections, compared with its

precursor - the cytoplasmic proteins of the epidermis - the characteristic feature of keratin is the higher concentration of cystine. The oxidative closure of sulphydryl groups to di-sulphide is also of considerable importance in the present study. The methods for sulphydryl and disulphides will therefore be discussed in much greater detail than those employed to demonstrate other substances.

Adult human skin used in this study was obtained from the Department of Plastic Surgery. It was removed generally from the thighs or lower abdomen, and placed into fixative within 15 minutes of removal. Details of fixation, tissue processing and staining methods are given in the appendix of technical methods (p.288).

2. Histochemical Demonstration of Sulphydryl and Disulphide Groups.

Four methods have been studied:

1. Red sulphydryl reagent (Bennett, 1951).
2. Ferricyanide (Chèvremont & Frederic, 1943).
3. Performic acid-alcian blue (Adams & Sloper, 1955 & 1956).
4. Dihydroxy-dinapthyl-disulphide (Barrnett & Seligman, 1952).

The first three methods proved unsuitable for the reasons given. That of Barrnett and Seligman (1952) was found to give satisfactory results and thereafter was employed routinely. It will therefore be considered in more detail than the others.

Red Sulphydryl Reagent (Bennett, 1951)

This method is based on the reaction of Mercaptans with 1-

(4-chloromercuriphenylazo)-2-napthol. With it there is the possibility of non-specific attachment to other protein groups, although, in practice, it is possible by using various SH blocking agents to demonstrate a high degree of specificity. This method was originally applied to teased tissue preparations and it has been found that the colour reaction in tissue sections is too faint for satisfactory localisation with the light microscope.

Ferric Ferricyanide (Chevremont & Frederic, 1943)

This method depends on the reduction of a fresh solution of fericyanide at pH 2.4 by SH groups. The resulting ferrocyanide combines with ferric iron to give a precipitate of Prussian Blue. Objections to this method include the possibility that groups other than SH may be oxidised. This can be overcome by adequate controls but in practice it renders the method cumbersome. Further, ferricyanide fails to oxidise all the SH groups and there is no well defined end point to the reaction.

Performic Acid - Alcian Blue (Adams & Sloper, 1955, 1956)

This method is probably the best of several which are based on the selective oxidation of cystine by performic acid. It includes the subsequent reaction of the sulphonic acid reaction product in an acid solution with the pthalocyanin dye, Alcian Blue. Cystine appears in varying shades of blue according to the concentration present.

Pearse (1960) states that the specificity of the method is high, but notes that the sensitivity is low and therefore demonstrates only high concentrations of cystine. This is in keeping with the findings in the present study. In addition, it has been found that there is a relatively limited range of intensity of staining rendering it difficult to do more than separate various areas broadly into 'high' and 'low' concentrations.

Dihydroxy-dinapthyl-disulphide (Barrnett & Seligman, 1952)

Rationale of the method: Barrnett and Seligman (1952) devised the above compound for the demonstration of sulphydryl groups. It contains a disulphide linkage (the specific oxidative group) and a napthol moiety to form an azo dye.

This reagent 22' dihydroxy-dinapthyl-66' disulphide (I), as shown in figure 3 (p.202) when used in excess at pH 8.5 reacts with the SH groups of fixed tissue proteins to form one protein napthyl sulphide (II) and one free napthyl mercaptan (III). A diazonium salt such as tetrazotised diorthoanisidine (Fast Blue B) combines with the napthyl moiety to form an azo dye (IV). The colourless oxidation product (II) is insoluble in both water and ether alcohol so that the excess of the reagent (I), as well as the reduced reaction by-products (III) can be washed out of the tissue with organic solvents.

Thus at the site of protein sulphydryl groups there is the rapid development of either a red or a blue colour. These colours

are interpreted by Barrnett and Seligman (1952) to indicate either monocoupling or dicoupling respectively, and the latter the higher concentration.

The Specificity of the Method: This depends on the specificity of the reaction of disulphide with sulphydryl groups at alkaline pH, as disulphides do not oxidise any groups in proteins other than sulphydryls.

The original authors state that it is not merely oxidation or reduction with transfer of electrons, but the napthyl moiety is transferred to the proteins and becomes part of the oxidation product, a protein napthyl sulphide, and because this compound is formed the reaction is specific. The specificity is proved by the loss of solubility in organic solvents of this napthyl moiety from which the pigment is produced.

Further proof of the specificity is the elimination of the reaction in tissues in which the SH groups are previously oxidised with iodine; subsequent treatment with a reducing agent restores the reaction, in addition to those groups obtained from naturally occurring SS groups. Pretreatment with specific blocking agents such as iodoacetate or N-ethyl maleimide also abolishes the reaction. It can also be prevented by incubating with excess sulphydryl (glutathione) before the reaction with the diazonium salt.

Pearse (1960) accepts the theoretical specificity of the method but notes that, in practice, a positive reaction may be obtained in sites

which are supposedly free from sulphydryl groups. Two possible explanations exist: (1) a diazonium reaction with histidine and tyrosine residues or (2) the incomplete extraction of excess DDD reagent or the reaction by-product (6 thio 2 napthyl). He suggests that the latter is the more important.

These findings have been confirmed in the present study. Thus initially in many of the sections of skin there was faint to moderate reaction in the dermis, presumably in the elastic tissue. Subsequently it was found that adequate washing in the organic solvents abolishes it, the times recommended by the original authors being the minimum possible. Details are given in the technical appendix (p.289). It is concluded therefore that, providing particular attention has been paid to this important technical point, the specificity of the method is high.

Method for the Demonstration of Disulphide Groups: The DDD method was subsequently modified by the original authors (Barrnett and Seligman, 1954) to demonstrate disulphide groups. This involves the irreversible blocking of the existing SH groups in the tissues and the reduction of the SS groups to SH.

In the present study blocking was achieved by 0.1 m. N-ethyl maleimide. This blocking was found to be unaffected by the relatively weak reducing agent potassium cyanide. Details are given in the technical appendix (p.290).

Distribution in Normal Adult Human Skin

Sulphydryl Groups: The reaction is almost completely confined to the cytoplasm of the cells. The only intranuclear structure which reacts at all, and then only faintly, is the nucleolus. The intensity of the staining in the basal and squamous layers is moderate and for the greater part uniform throughout. Superficially the squamous layer, at its junction with the keratin layer, shows a zone of moderately increased intensity of staining. This zone includes the Stratum Granulosum but the keratohyalin granules are not reactive. The keratin layer is much less reactive. An approximation of the intensity of the staining might be 50% that of the squamous layers (figure 4, p.203).

Disulphide Groups: These groups give an extremely faint reaction in the basal and squamous layers. The reaction is uniform throughout, there being no intensification in the Stratum Granulosum. Again the keratohyalin granules are completely unreactive. The staining in the keratin layer is much more intense and in many instances it is the only structure which is visible (figure 5, p.203).

These findings are in general agreement with those of Barrnett and Seligman (1954), who studied rat skin and Montagna et al. (1954) who investigated human material. They do not confirm the findings of Giroud and Leblond (1951), who claim that the intensification of the sulphydryl reaction in what they described as the "kerato-

genous zone" is only present in the so-called "hard" keratins such as nails or hair, and does not occur in "soft" keratin structures such as skin. They are also not in agreement with Van Scott and Flesch (1954) who found the concentration of disulphide groups was the same in the squamous and keratin layers, and that the concentration of the sulphydryl groups in the squamous layer was relatively small and became even smaller during keratinisation.

3. Other Histochemical Methods.

Glycogen: Although squamous epithelium elsewhere in the body often contains abundant glycogen, human skin contains relatively little (Wislocki et al., 1951). The methods of fixation and processing influence the amount demonstrable. Thus, much higher concentrations of glycogen in various sites have been demonstrated when the tissues have been fixed by the technique of freeze drying (Mancini, 1948). Again the amount demonstrable varies with the method used. Throughout the present study the method used is that of the Periodic acid-Schiff (Appendix, p.291). Control sections are stained after treatment with saliva or 1% diastase for 30 minutes. The specificity of this method has been discussed at length by Pearse (1960) who considers that there is no doubt that glycogen present in fixed sections is always entirely removable by saliva or diastase. Therefore in the subsequent descriptions the term glycogen refers to Schiff positive material which is removable by

diastase or saliva.

In normal human epidermis the amount of glycogen demonstrable by this method is scanty and distributed irregularly. Thus, it is completely absent in the basal and keratin layers, apart from the basement membrane at the dermo-epidermal junction. When present in the squamous layer it is restricted to the outer half of this layer and it tends to be present in groups of adjacent cells. It is again more abundant around the orifices of the pilo-sebaceous follicles. It is extremely scanty in Stratum Granulosum and it is not demonstrable in the keratohyalin granules.

Deoxyribonucleic Acid: The Feulgen reaction (Feulgen & Rossenbeck, 1924) was used throughout. In this reaction Schiff's reagent (Fuchsin sulphurous acid) reacts with aldehyde groups released from the deoxypentose sugar of DNA by mild acid hydrolysis, and DNA is demonstrable as purple coloured material.

Again the specificity is discussed at length by Pearse (1960), and subsequently the purple material which remains after this reaction is regarded as DNA.

It is normally confined to the nucleus and in the basal and lower squamous cells it is present as fine purple granules of uniform size. As the squamous cells approach the Stratum Granulosum there is a rapid but uniform condensation of the nuclei until they appear as tiny solid masses of DNA. The keratohyalin granules are completely unreactive with this method.

Ribonucleic Acid: The method used (Brachet, 1942) depends on the specific depolymerisation of ribonucleic acid (RNA) by the enzyme ribonuclease and the subsequent red staining with a solution of pyronin-methyl green. Material which is removable by ribonuclease is considered to be RNA. This method was found to give relatively poor cytological detail. The reaction is confined to the cytoplasm with the exception of the nucleoli which generally stain with moderate intensity. The cells of the basal and lower squamous layers show the highest concentration and there is a gradual diminution in staining until the lowest level is reached in the granular layer. The keratohyalin granules are found to be uniformly unreactive with this method.

Alkaline Phosphatase: The Azo coupling technique as modified by Pearse (1960) was utilised. Staining is confined to the capillaries of the dermal papillae. No enzyme is demonstrable in the epidermis and, in particular, the keratohyalin granules are completely unreactive.

Acid Phosphatase: Holt's modification of Gomori's method was used (Holt, 1959). By this method the precipitate forms a dense band at the junction of the squamous and keratin layers. Precise localisation at this level is very difficult but the keratohyalin granules, where they can be positively identified, are unreactive (figure 6, p. 204).

CONCLUSIONS

The keratins are a widely occurring group of substances whose main function is of a protective nature. As has been illustrated, they have a variable amino-acid composition. This holds between different species and between keratins from different sites in the same species. In view of this it is, perhaps, rather surprising that attempts have been made to classify the various types of keratin on the basis of their chemical composition. Thus Block (1939), on the basis of the amino-acid molecular ratio, classified human epidermal keratin as a pseudo-keratin along with tortoise-shell. Giroud et al. (1934), on the basis of a combination of chemical and physical properties, separated keratins into hard and soft, epidermal keratin being in the latter category.

Although in general these classifications may be of some value, they do not stand up to critical analysis. Thus in the previous section it has been shown that in human skin (a 'soft' keratin structure) there is an intensification of the sulphydryl staining at the junction of the squamous and keratin layers. This so-called "keratogenous zone of Giroud" was described by him as only occurring in hard keratins. In any event, it is probable that Rothman (1954) is correct when he states "keratinisation is a progressive process and thus it is the degree of keratinisation which determines how the end product will behave".

Again the analyses of the precise chemical composition of the keratins has yielded widely varying results. Overall, the data on keratins reproduced from Block (1951) appears to have gained most general acceptance. More recently Muting et al. (1955) have produced essentially similar data with particular respect to the amino-acid composition of the human epidermis and human epidermal keratin. Their findings have been criticised by Flesch (1958) on the grounds that cystine values are surprisingly high. The same author, however, concludes despite this criticism that a variable and occasionally inconstant rise in cystine is the only definitely established change in the course of epidermal keratinisation.

Lustig et al. (1958) have also analysed the chemical composition of keratins from skin diseases such as psoriasis, exfoliative dermatitis and disseminated neurodermatitis. Although their values again were lower, there was still a rise in various amino-acids and particularly cystine, when they were compared with those of epidermis. They found no difference in the amino-acid composition in the keratin from the various disorders.

It appears, therefore, that there is no good evidence against the statement that, although keratins show a fairly wide variation in composition, the one chemical feature which is constantly present is a variable but higher concentration of cystine compared with a precursor such as the epidermis.

Little definite evidence exists on the precise nature of this chemical change. Thus Flesch (1958) has postulated that keratinisation is a two step process, the first being the formation of a fibrous precursor in the epidermis with little or no sulphur. This precursor then combines with sulphur containing proteins to form the consolidated keratin.

The nature of this fibrous precursor is doubtful. However, Rudall (1946) has extracted from the cow's nose a fibrous protein substance which he called epidermin. Roe (1956, a and b) in extraction studies on human skin with lithium bromide found that a substance could be extracted which after stretching showed an α X-ray diffraction pattern. However, there is still no definite evidence to disprove the widely accepted and attractive theory that the final consolidation of the keratin molecule takes place by the oxidative closure of adjacent sulphydryl groups to form disulphide cross linkages.

The points in favour of the specificity of the dihydroxy-dinapthyl-disulphide stain for demonstrating such linkages have already been discussed. It is considered to be the best stain available at the moment for this purpose and, if attention is paid to technical detail, its specificity is high, giving consistently reliable results. It is difficult to explain the presence of SH groups in addition to SS in the keratin layer, but it is believed

that their presence in both the squamous and the keratin layers does not invalidate in any way the contention that oxidation of these groups is an essential part of the keratinisation process. They are undoubtedly reduced in amount and the fact that disulphide has only been shown in the material which is generally accepted as keratin, would give further support to this view. The presence of the increase in SH groups in the zone of transition is also difficult to explain. It may be that at the time of keratinisation there is a hydrolytic splitting off of amino-acids which do not contain sulphur with a relative increase of those which do contain sulphur. In any event, it in no way detracts from the hypothesis.

From the earlier descriptions about what is known about the chemistry of keratinisation it is not surprising that the other histochemical methods have yielded little of direct value. It is important to remember, as has been pointed out by Rothman (1954), that the terms keratin and keratinous structures are not identical. Again, it is relevant that the chemical analyses of keratins have been carried out on purified material in that lipids and other substances have been previously removed. A similar state of affairs exists in sections of skin which have been processed in various solvents and embedded in paraffin wax. Thus, when thinking about keratinisation, one may tend to forget that the keratin layer (S. Corneum) contains substances other than keratin.

Thus, as Flesch (1958) points out, the water soluble components,

which amongst others include carbohydrates, urea and free amino-acids, are essential for the normal shedding process of keratin. If their metabolism is disturbed either by a disease such as psoriasis or from exposure to physical agents such as excessive cold or chemical agents such as detergents, scaling and other abnormalities may result.

The source of these substances is, of course, not solely confined to the epidermis. Thus in a detailed study by gas chromatography of the surface lipids, Reinertson and Wheatley (1959) have shown that these lipids consist of a mixture of sebaceous gland products and of lipid from the keratinising epidermis.

The presence of such lipids may explain the initially puzzling finding of the high concentration of acid phosphatase in the intermediate zone between the squamous and keratin layers. It is however probable that the breakdown of phospholipids as undoubtedly occurs in this region (Reinertson & Wheatley, 1959) is dependent upon this enzyme.

In summary the other histochemical methods showed only those findings which might be expected with the accompanying degeneration of the cell.

A constant feature in normal skin at the transition zone between the squamous and keratin layers is the presence of keratohyalin granules. As has been already stated, on morphological grounds they have been implicated on numerous occasions as keratin

precursors but there is really little definite evidence to support this view. Recently Brody (1959), in an electron microscopic study of these structures in the normal epidermis of the guinea-pig, claimed that tonofilament bundles in this layer are oriented around the granules and again suggests that they represent a precursor state in the keratin pattern. The evidence for this is probably inconclusive. Selby (1957) using human skin could not demonstrate any such relationship in an earlier study. The present findings do not offer any fresh evidence but the complete absence of any reaction in the granules for either sulphydryl and disulphide groups strongly suggests that they are not directly involved in the process of keratinisation and are, in fact, probably degenerative "by-products".

On the purely morphological aspects of keratinisation, as manifest by the usual Haematoxylin and Eosin stained sections, this survey has little fresh to add. The tonofibrils of classical histology which were thought to be the basis of the process of keratinisation were not seen with any degree of regularity. They were so randomly distributed that one must at least, as far as the light microscopy goes, agree with Medawar (1953) who stated that tonofibrils have most of the stigmata of artefacts. He continues, however, "that possibly all histological appearances are artefacts in the sense that they represent, as a result of technical processing, a coarsening of the submicroscopic living system to the level

of microscopical visibility".

However, electron microscopic studies have extended the morphological approach to this submicroscopic living system. Various types of epithelia have been studied (Porter, 1954; Weiss & Ferris, 1954; Odland, 1958), and structures which have been described as tonofibrils can be demonstrated. Their precise role in the process of keratinisation, however, still remains to be elucidated.

Thus Swanbeck (1959) has recently made further X-ray diffraction studies of human material and found that the fibrils of the squamous layer are scattered independently and they measure 260A. He suggests that the aggregation of seven tonofibrils into this 260A fibril is the main step in the process of keratinisation and that this would fit with the molecular model which was proposed by Pauling and Corey (1953). He does, however, stress that he puts this theory forward on a purely speculative basis. Thus the basic process of keratinisation although no doubt nearer solution, remains unsolved.

SUMMARY

Keratins are fibrous proteins which are widely distributed and are characterised by a variable amino-acid composition. From a survey of the relevant literature, it emerges that there is only one constant chemical feature which characterises the keratins as compared with other proteins, namely a high content of cystine. Further, in the process of keratinisation there are only two changes which have been demonstrated with any degree of constancy, a disappearance of hydroxyproline and a variable rise in cystine. The largest part of the cystine sulphur is present in the form of disulphide cross linkages. There is no satisfactory evidence to disprove the reasonable hypothesis that during the process of keratinisation the final consolidation of the molecule is brought about by the oxidative closure of sulphydryl containing amino-acid residues on neighbouring polypeptide chains, thus forming disulphide cross linkages.

The standard methods for the histochemical demonstration of these groups have been applied to normal human adult skin. Of these only the dihydroxy-dinapthyl-disulphide method has proved to be satisfactory, both as regards theoretical specificity and the consistent production of comparable findings. With this method it has been shown that sulphydryl groups are present in the squamous cell layer, and, although in considerably lower concentration, are

also present in the keratin layer. There is a marked increase in concentration at the junction of these two layers. The keratohyalin granules are unreactive and are considered to be products of the degenerating cell. Disulphide groups are only demonstrable in the keratin layer. These findings are considered to give support to the currently accepted hypothesis of the mechanism of the keratinisation process. The other methods employed have not contributed anything further to a study of this process. The findings are, however, of importance as standards for comparison with those in abnormal keratinisation.

P A R T II

ABNORMAL KERATINISATION IN HUMAN SKIN

INTRODUCTION

Abnormal keratinisation of squamous epithelium is generally present in one of two ways.

1. Hyperkeratosis where there is excessive thickness of the S. corneum. This is usually associated with hyperplasia of the squamous layer.
2. Dyskeratosis where there is abnormal keratinisation of individual cells which often show premature keratinisation within the squamous layer.

Often both types are present in the same lesion and they occur in many skin diseases. Hyperkeratosis is commonly found in various reactive states, such as psoriasis or lichen planus, which are characterised by hyperplasia of the epidermis. It is also frequently seen in hyperplastic conditions such as senile keratosis. Dyskeratosis is usually divided into two types - benign dyskeratosis, such as is seen in Darier's disease, and malignant dyskeratosis, e.g., intraepidermal carcinoma.

A common lesion which provides a good model for a study of abnormal keratinisation in human skin is the simple squamous papilloma - verruca vulgaris. Through the co-operation of Dr. T. Cochrane, it was possible to study large numbers of these lesions in considerable detail. Although all were considered clinically to

be verrucae vulgares, two histologically distinct types were found and each showed abnormal keratin formation. In one group, the production is excessive but still orderly, i.e., hyperkeratosis. In the other, while excessive keratin is produced, the process is much less regular and shows keratinisation of individual cells, deep in the squamous layer, i.e., an excellent example of dyskeratosis.

In addition, the two types show distinctive clinical features. The 'dyskeratotic' type is also associated with intranuclear 'inclusion' bodies.

CLINICAL FEATURES OF VERRUCAE VULGARES

Four hundred warts were examined from 164 patients and, as can be seen (Table III, p.177) clinically the lesions fall into two main groups. Those of the larger group are histologically hyperkeratotic papillomata and subsequently they will be referred to as the hyperkeratotic group. Similarly the other main group will be referred to as dyskeratotic. The main clinical features of both types are given in Table IV (p.178). The hyperkeratotic lesions are commonly found on the fingers, face and limbs. They are superficial and present as irregular excrescences (figure 7, p.205). The dyskeratotic type occurs mainly on the palmar aspect. They are discrete, deep seated with a domed top and are surrounded by a hyperkeratotic collar (figure 8, p.205). Other differences are also shown in Table IV (p.178). In the hyperkeratotic group 66 of the patients are under 20 years of age, while a larger percentage are above this age in the dyskeratotic group. In the hyperkeratotic group over half of the patients have more than 7 warts and very large numbers are present in many of them. Only 2 have more than 6 lesions in the dyskeratotic type. Many of the patients are certain of the source of infection in the dyskeratotic group. On the other hand, only a few are certain in the hyperkeratotic group and the dermatologist is doubtful of most of these. The main clinical features are summarised in Table V (p.179).

THE HISTOPATHOLOGY OF VERRUCAE VULGARES

In addition to the clinical differences there are distinctive histopathological features which readily permit their classification into hyperkeratotic and dyskeratotic groups.

A. HYPERKERATOTIC PAPILLOMATA

There is considerable increase in the thickness of the squamous cell layer (acanthosis). The interpapillary processes are broadened and elongated and often enclose portions of the dermal papillae. Superficially there is a dense broad layer of keratin (figure 9, p. 206). At higher magnification the squamous cells are similar to those of normal skin. Their polarity is retained and intercellular bridges are prominent.

Several features are frequently but not invariably present. Thus the cytoplasm of some cells is perhaps more basophilic and granular than in normal skin. The nuclei again differ slightly. The nuclear membrane is often more prominent and the chromatin less dense. Although mitotic figures are very infrequent, there is slight pleomorphism. Basophilic or faintly eosinophilic nucleoli are usually present.

A frequent finding is the presence of large vacuolated cells in the upper squamous layer. The intercellular bridges between such cells are lost and there appears to be some condensation of cytoplasmic material into faintly eosinophilic strands, some of

which are slightly refractile. These may represent 'tonofibrils'. The nuclei which are initially swollen, gradually disintegrate and in the granular layer form dense collections of deeply basophilic material (figure 10, p. 207). In common with many other hyperkeratotic lesions of skin there is often considerable thickening of the granular layer where the keratohyalin granules are large and deeply staining (figure 11, p. 208). The keratin layer is greatly thickened and consists of densely eosinophilic material in which there are occasional parakeratotic cells (figure 12, p. 208).

Thus although slight cytological differences exist between these lesions and normal skin, only three features are present regularly and could be considered striking, i.e., pronounced acanthosis with hyperkeratosis, a prominent granular layer and vacuolisation of cells in the upper squamous layer. Compared with normal skin the appearances suggest an excessive and possibly accelerated production of keratin. The process however, is still essentially integrated and orderly.

B. DYSKERATOTIC PAPILLOMATA

These lesions also are characterised by considerable hyperplasia of the squamous cell layer with excessive production of keratin. The process, however is a disorderly one and bears little resemblance to that in normal skin. This lack of order is suggested even on low power examination of the lesions. They

show irregular acanthosis and the squamous layer forms a series of papillary processes which are separated by masses of keratin-like material (figure 13, p. 209).

The lesions also contain numerous dyskeratotic cells at various stages of development throughout the epidermis. The affected cells are first recognisable low in the squamous cell layer (figure 14, p. 210), where isolated cells contain multiple tiny solid particles in their cytoplasm. These are deeply eosinophilic and the average number is about 20 per cell. As they approach the surface the particles coalesce to form large collections of hyaline eosinophilic material (figure 15, p. 211), which merge to form keratin in which there are swollen nuclei (figure 16, p. 212). Superficially this material becomes less deeply eosinophilic and more refractile. Nuclear debris is often present high in the keratin layer. Basophilic keratohyalin granules are not a prominent feature and where present are confined to those areas in the lesion where groups of 'unaffected' cells are present, i.e., where the process of keratinisation more closely approximates to that of normal skin (figure 17, p. 213).

In addition, high power examination of the dyskeratotic cells shows that they contain intranuclear 'inclusion' bodies. These bodies are single, and markedly eosinophilic (figure 18, p. 214). They are first seen deep in the squamous layer where they occur

in isolated cells (figure 19, p. 215). The average size of the bodies in the lower layers is about 10 μ and they gradually increase in size as they progress towards the keratin layer. Here, only a few bodies can be identified in swollen nuclei of which they may occupy one quarter. The nuclei of the affected cells, in the lower squamous layer appear little different from those surrounding them, apart from the presence of the inclusion body. Basophilic or faintly eosinophilic nucleoli are also clearly seen in many of the affected cells. Some cells show slight but distinct margination of the basichromatin at the edge of the nucleus. Mitotic figures are not seen in any of these cells while they are not infrequent in the adjacent unaffected cells.

The whole process of keratinisation in these lesions appears to be proceeding more rapidly than in either normal skin or the hyperkeratotic lesions. It is also uncoordinated in that individual squamous cells show varying degrees of keratinisation which are far in advance of their neighbours.

HISTOCHEMISTRY

A. INTRODUCTION

Both types of lesion have been examined by the methods described in Part I (p. 9). As will be shown, there is relatively little difference histochemically between the two lesions or between them and normal skin. The only distinctive histochemical findings are in relation to the concentration and distribution of sulphydryl and disulphide groups. Accordingly the results obtained with the other methods will be described briefly and those concerning the sulphydryl and disulphide groups considered in greater detail.

B. SULPHYDRYL AND DISULPHIDE GROUPS

Sulphydryl: A positive reaction is found in the cytoplasm of basal and squamous cells of both groups. The only intranuclear structure uniformly reactive is the nucleolus. In the dyskeratotic lesions the intranuclear inclusion bodies give a variable reaction, but in most instances this is negative, especially when they become larger in the more superficial layers.

In the hyperkeratotic lesions staining is uniform and of moderate intensity throughout the squamous and basal layers. The keratin layer is less reactive. A broad band of intense staining, however, is present at the junction of the squamous cell

layer and the keratin layer (figure 20, p. 216). This zone corresponds to the granular layer and the area immediately above it. With the exception of an occasional doubtful reaction the keratohyalin granules are negative and even where doubtful it is impossible to be certain that it is not the background of the cytoplasm which is reactive. The reaction for sulphydryl extends far out into the keratin layer but gradually becomes less intense and is virtually absent in the outermost layers (S. Disjunction). The process as illustrated by this method, resembles that of normal skin and is essentially orderly and coordinated.

This is in contrast to the dyskeratotic lesions. In general, the squamous layer reacts uniformly and to the same degree of intensity as in the hyperkeratotic lesions, with the exception of the groups of dyskeratotic cells which react intensely. The reactive substance in these cells corresponds in size and distribution to the deeply eosinophilic material seen in the sections stained by haematoxylin and eosin. It first appears in the depths of the squamous cell layer, as small round droplets which enlarge and assume bizarre shapes as the surface is approached. They gradually coalesce and merge into deeply staining masses of acellular material which form finger-like processes between which are groups of hyperplastic 'unaffected' cells (figure 21, p. 216). The overlying keratin layer is moderately reactive, staining to the same intensity as the greater part of the squamous cell layer.

There is no definite transition zone of strongly reactive material as occurs in normal skin and is a pronounced feature in the hyperkeratotic lesions. More deeply staining material is seen however in some areas and this extends high into the keratin layer in places, (figure 22, p. 217).

Disulphides: In the hyperkeratotic lesions the reaction is very weak in the squamous cell layer and apparently confined to the cytoplasm. Keratohyalin granules again fail to react. The keratin however stains intensely (figure 23, p. 218).

In the dyskeratotic lesions the reaction is positive in the keratin layer. The cytoplasmic material also reacts and the intensity of staining parallels the development of this material (figure 24, p. 218). Thus in the lower layers where only isolated cells are affected, the small granules are unreactive. As the masses become larger and those in adjacent cells coalesce, the staining gradually increases in intensity until in the uppermost layer there are irregular masses of material, all of which exceed in intensity the staining seen elsewhere, including even the apparently fully developed keratin layer.

C. OTHER HISTOCHEMICAL METHODS

Nucleic Acids: Examination by the Feulgen method for DNA reveals little difference between normal skin and the hyperkeratotic type of wart. The staining is confined to the nucleus, and the only difference is that the chromatin in the hyperkeratotic lesions is arranged in clumps which are larger, more irregular in size and stain more densely.

In the dyskeratotic lesions staining is again confined to the nucleus. The eosinophilic cytoplasmic material throughout all stages of its development, and the fully developed keratin, stain with the green counterstain. The nuclei show greater variation in the size and arrangement of the Feulgen positive chromatin. This becomes much more pronounced in the more superficial layers where fragmentation of the nucleus can be seen frequently and large Feulgen positive fragments are scattered throughout the keratinised material. There is no evidence of DNA in the nucleoli or inclusion bodies. Both these structures stain with the green counterstain, the latter however often only faintly.

In the hypertrophic type the RNA is again confined to the cytoplasm with the exception of the nucleolus. Compared with normal skin it is slightly increased in amount. The greatest concentration is in the lower squamous layer and it gradually diminishes as the keratin layer is reached. The keratohyalin

granules are again found to be unreactive.

The dyskeratotic warts consistently show considerably higher concentrations of RNA. This is, however, restricted to the 'unaffected' cells, i.e., those cells which contain neither inclusion bodies nor cytoplasmic masses. Groups of these cells containing fairly high concentrations of RNA alternate with groups of affected cells which are almost completely unreactive.

The cytoplasmic masses occasionally show slight staining. This reaction is, however, not abolished by ribonuclease. The keratohyalin granules are again found to be consistently unreactive. In the nuclei the only structures which stain are the nucleoli. The inclusion bodies appear as unstained spaces which are surrounded by a halo of condensed chromatin.

Glycogen: The hyperplastic epithelium of both types shows only a slight increase in the amount of glycogen. This occurs as fine droplets of P.A.S. positive material scattered diffusely throughout the cytoplasm. In the hyperkeratotic type it is present in the lower and middle thirds of the squamous cell layer and distributed evenly throughout. In the dyskeratotic type, on the other hand, it tends to occur in the lower squamous layer. It is found in groups of neighbouring squamous cells, the intervening groups being unreactive. The latter are those which have inclusion bodies and are developing intracytoplasmic masses. In both types the

upper squamous and keratin layers show no evidence of glycogen, and the keratohyalin granules are unreactive.

In the dyskeratotic type neither the cytoplasmic masses nor the intranuclear inclusion bodies show any histochemical evidence of glycogen.

Lipids: The Sudan black B stain for total lipids yields only negative results in both types of lesion. In particular, the granular layer is completely unreactive.

Enzymes:

Alkaline phosphatase: This is confined to the capillaries of the adjacent dermal papillae. No activity is demonstrable in hyperplastic squamous epithelium of either type of wart. Again the keratohyalin granules are completely unreactive.

Acid phosphatase: In both types of lesion the hyperplastic epithelial layer and keratin are completely unreactive. In particular, the transitional zone between these two layers does not stain, although there is an intense band of staining in the corresponding zone in the adjacent normal squamous epithelium.

DISCUSSION

Although often referred to as such, warts are not true tumours (Willis, 1960). They are excluded by definition, since, in time, they probably all resolve spontaneously.

Their infective nature is well established and, although Koch's postulates have not been satisfactorily fulfilled, there is good evidence that a virus is the responsible agent. This evidence is briefly reviewed in the introduction to Part III. A discussion of the nature of inclusion bodies is outwith the scope of the present study; nevertheless it is relevant that whatever their origin or chemical nature, they are frequently the morphological stigmata of a virus infection. While inclusion bodies have been described in warts by various observers (Dubreuilh, 1895; Sangiorgi, 1915), many authors still regard them as degeneration products (McCarthy, 1931; Percival et al., 1947).

More recently Lyell and Miles (1951) together with Strauss et al. (1950) claim that the intranuclear eosinophilic bodies, originally described by Dubreuilh (1895), are inclusion bodies which occur in a clinically distinct type of wart. Blank et al. (1951) however, think that these bodies are usually active nucleoli and claim that deeply basophilic nuclei which occur in the upper squamous cell layer and granular layer are filled with virus inclusion material.

The present findings support and extend those of Lyell and Miles and Strauss and his colleagues (1950). It is believed the common infective lesions clinically acceptable as warts, fall into two main groups which can be distinguished on clinical and histological grounds. The less common lesion is usually single, deep seated and present on the palmar or plantar surfaces. It is characterised histologically by the presence of an intranuclear inclusion body and distinctive morphological features including the presence of numerous dyskeratotic cells.

The suggestion by Blank et al. (1951) that the intranuclear bodies are unusually active nucleoli must be considered, as it is well recognised that in neoplastic and reactive conditions the nucleoli are often more prominent than in normal tissues. Thus the relative size of the nucleolus to the nucleus has been suggested and used as a method for the histological grading of malignancy (Naidu, 1935; Ferreira, 1941).

However, that these intranuclear bodies are not degeneration products or nucleoli is suggested by the fact that they possess distinctive morphological and histochemical features. They are not seen in any other condition characterised by hyperplasia of squamous epithelium. They do not occur in every cell and are frequently seen in cells only one layer above the basal cell layer. The cells otherwise show no evidence of degeneration and may thus escape the criticism of Ludford (1930) who, describing the Stratum

Granulosum of skin, said "in this situation the cells are degenerating and all kinds of granules can be stained, offering a wide choice to seekers after virus bodies".

Deeply basophilic bodies which have all the morphological characteristics of nucleoli can frequently be seen in the same nucleus. In those sections stained by pyronin-methyl green, the nucleoli are positive (the reaction being abolished by ribonuclease) and the inclusion bodies are not. With prolonged differentiation in the phloxine tartrazine method (Lendrum, 1947) the bodies are the only structures which remain stained.

It seems reasonable, therefore, to conclude that whatever their nature or source, these bodies are not nucleoli. Further, they are a diagnostic feature of a clinically distinctive type of wart.

It may be significant that the distribution of these dyskeratotic inclusion bearing warts is mainly on palmar or plantar surfaces. Thus pressure may be cited as a factor particularly in the latter site. While this cannot be excluded completely, it is obvious on clinical grounds that these lesions occur often when pressure is unlikely to be a factor. Again, the hyperkeratotic type does occur in sites where pressure on the lesions undoubtedly occurs.

The role pressure may play in modifying the clinical and morphological appearances leads on to the question, are these two

distinct lesions or are they different reactions to the same infective agent? This cannot be answered at present because the infective agent or agents have not been isolated either on the chorio-allantoic membrane of the chick (Fischer, 1953; Pullar & Cochrane, 1957) or in tissue culture of hela cells (Siegel & Novy, 1955) or human amnion cells (Pullar: unpublished data). Again, although infective papillomata are common in animals, they appear to be species specific and there is no convincing evidence of the successful transmission of human warts to animals. The only record of transmission experiments in human volunteers (Lyell & Miles, 1951) suggests that they are two distinct lesions, but the evidence for this is not altogether convincing.

Whatever the explanation, it remains that these infective lesions have distinctive clinical and morphological features. Again, there is a difference in the way in which they become keratinised. Compared with normal skin a constant feature is a considerable increase in the amount of keratin, that is, both show hyperkeratosis. In those referred to as the hyperkeratotic group, this is pronounced. The appearances, however, suggest an accelerated but still orderly process. In the dyskeratotic group there is again considerable increase in the amount of keratin formed. The most striking feature, however, is the keratinisation of individual cells (dyskeratosis). As has been described, all stages in this process

can be traced from the first appearance of tiny eosinophilic droplets in single isolated cells deep in the squamous cell layer to the final incorporation of large masses of keratin-like material in the superficial keratin layer. The histological appearances suggest that this is a much more active lesion and that the processes of keratinisation and cell destruction are also proceeding much more rapidly.

As stated above the hyperkeratotic type on morphological grounds appears to show an acceleration of the normal process of keratinisation. The histochemical findings are in keeping with this impression. DNA is shown to be irregularly distributed and increased in amount. The changes, however, are relatively slight, and there is no evidence of the Feulgen positive inclusion bodies which Blank et al. (1951) described in the upper squamous and granular layers. It is distributed much more irregularly in the dyskeratotic lesions. This is particularly evident in the partially keratinised areas where there are dense collections of Feulgen positive material presumably nuclear fragments showing varying degrees of degeneration.

In addition, as these lesions are probably virus induced, their DNA content is of considerable importance. In the present study it has not been possible to demonstrate conclusively the presence of DNA at any stage of development of the inclusion body.

The DNA content of nuclei of warts has however been studied by more sensitive microphotometric techniques by Bloch and Godman (1957). They found in warts of the dyskeratotic type increased amounts of DNA (up to 16 ploid levels) appearing in the nucleus at the earliest recognisable stage of infection. There appears however, to be no direct relationship between the Feulgen positive DNA and keratin at any stage of its development.

The changes in the RNA content in the hypertrophic lesion, although slight, are consistently present. There is moderate increase in the basal and lower squamous layers. Although the concentration is higher than that of normal skin, it does show a similar distribution pattern in that it gradually diminishes in the more superficial layers, and is no longer demonstrable by this method in the granular or keratin layers.

Again this inverse relationship between the amount of RNA and degree of keratinisation is even more obvious in the dyskeratotic lesions where, in the lower squamous layer, groups of dyskeratotic cells alternate with unaffected cells. The latter, contain relatively high concentrations of RNA while it is almost completely absent from the partially keratinised dyskeratotic cells. This is as might be expected, considering how intimately RNA is concerned with cell metabolism. Although part of the function of the metabolism of the squamous cells may be concerned with the formation of the keratin precursor, keratin is a completely devitalised

structure.

These findings are in accord with those described in reactive skin after injury (Washburn, 1954a) and in experimental carcinogenesis of skin (Biesele, 1944).

The results obtained with both acid and alkaline phosphatases suggest that neither is directly involved in the process of keratinisation. Thus intense acid phosphatase staining can be demonstrated in the granular layer but not in the hyperplastic keratinising lesions of either type. This is taken as support to the view that this enzyme is probably concerned with lipid metabolism, the lipids being mainly derived from the normally functioning appendages in the adjacent skin.

The present findings confirm the findings of Nadel and Wodinsky (1955), who suggested that earlier reports (Fell & Danielli, 1943; Fisher & Glick, 1947) of alkaline phosphatase activity in the S. granulosum were probably the result of diffusion with subsequent adsorption on the keratohyalin granules. It is concluded that at present there is no histochemical evidence that alkaline phosphatase is directly concerned with the process of keratinisation.

In the hyperkeratotic type glycogen is increased both as regards the number of cells in which it is demonstrable and the amount in these cells. It is present only in those cells which show no morphological evidence of keratinisation. Thus it is present in

inverse proportion to the degree of keratinisation. This inverse relationship is also found in the dyskeratotic lesions where a moderate amount of glycogen is present in the groups of unaffected cells. Their neighbours, which may show only the earliest changes of individual cell keratinisation, are completely devoid of this material.

This inverse relationship has been established for over a century. Claude Bernard (1859) observed in the hoof of the pig that glycogen disappears at the onset of "organisation" (keratinisation). It has also been confirmed in regenerating epidermis in the experimental animal (Bradfield, 1951; Washburn, 1954b).

The precise nature of this relationship of glycogen to keratinisation, however, remains unproved. Normally epithelial cells require glycogen for both aerobic and anaerobic glycolysis (Berenblum et al., 1940). Bradfield (1951) suggested that the accumulation of glycogen in the epithelium in healing wounds resulted from the poor supply of glucose and oxygen. Medawar (1947), who maintained skin alive for 1 week under strictly anaerobic conditions, also found that exposure to iodo-acetate caused death and suggested that it was the breakdown of sugar which provided the energy for survival. Cellular accumulation of glycogen may be a degenerative phenomenon as suggested by Scothorne, &

Tough (1952) in a study of the content of glycogen in human skin autografts and homografts. As they point out, however, this explanation is not altogether adequate as the process must involve synthesis by the cell. Again, as discussed by Dempsey and Wislocki (1944), it may represent an adaptation to conditions of reduced oxygen tension. The basis of this theory is that cells living under such conditions may adopt anaerobic methods of carbohydrate breakdown. Since these are less efficient proportionately larger quantities of substrate will be required to furnish the same amount of energy, the energy presumably being used for protein synthesis in keratinisation. Whatever the precise mechanism involved, it remains that this inverse relationship is also present in abnormal keratinisation and in both types of lesion. The observations suggest that mitotically active cells and cells in the process of accelerated keratinisation utilise glycogen but do not accumulate it.

The distribution of sulphydryl and disulphide groups in the hyperkeratotic lesions would appear to support the contention that this type of lesion represents an accelerated rate of keratin formation but the process is essentially still an orderly one. Thus the distribution of the disulphide is still restricted to that part of the S. corneum which morphologically is acellular and composed of refractile fibres. Although the distribution and

relative concentration of sulphydryl are essentially similar to those of normal skin, there are two features worthy of comment. Firstly, although the concentration in the keratin layer is lower than that of the squamous layer, the amount of the reduction is not so pronounced as in normal skin. Secondly there is a considerably wider band of intense staining interposed between the keratin and squamous layers. Why this increased concentration of SH groups should be present in this zone of transition is a matter for speculation. As already suggested in Part I it probably represents the area in which the keratinising cells lose most of their water and where there is a hydrolytic splitting off of free amino acids with a consequent relative increase in the amount of demonstrable sulphydryl groups. The extension of this zone in more rapidly growing lesions may result from an acceleration of this process, and the SH containing amino acids are carried far into the keratin zone before there has been time for complete consolidation to take place.

In the dyskeratotic lesions the findings are essentially those of rapid and complete keratinisation of individual cells. The changes which are present in skin, and, in a similar, although exaggerated form in the hyperkeratotic lesions, are exhibited at cellular level. Thus very shortly after the eosinophilic masses become visible they show a moderate reaction for sulphydryl, and as

they become larger this increases. This higher concentration extends well into the keratin layer. Again in sections stained for disulphide these groups can be demonstrated in affected cells low in the squamous cell layer.

The findings show that the sequence of events in both types of abnormal keratinisation is similar to that found in normal skin. The same sequence is found whether the process occurs in single isolated cells in the dyskeratotic lesions or in the hyperkeratotic lesions where it represents an accelerated but co-ordinated stage in the development of squamous epithelium.

SUMMARY

The morphology and histochemistry of abnormal keratinisation have been studied in human infective warts. The lesions which morphologically are squamous papillomata can be classified on clinical and histological grounds in two main groups. Both show different forms of abnormal keratinisation. The more common lesion, which is referred to as Hyperkeratotic shows an excessive and accelerated, but still orderly process. In the other main group, the Dyskeratotic, the process is completely unco-ordinated, and all stages can be demonstrated in individual cells. Histochemical studies in these lesions show that there is an inverse relationship between the amount of demonstrable glycogen and the degree of keratinisation. The distribution of sulphydryl and disulphide suggests that there is no essential difference in the mechanism of keratin formation as it occurs in these abnormal lesions. In both types there is an acceleration of the process which is similar to that which occurs in normal skin.

PART III

EXPERIMENTAL STUDIES ON THE CHICK CHORIO-ALLANTOIC MEMBRANE

INTRODUCTION

The examination of a series of skin papillomata has shown that two clinical varieties exist and are associated with constant and distinctive histological features (Pullar & Cochrane, 1957). One of these varieties shows mainly pronounced hyperkeratosis and the other dyskeratosis. Many of the cells of the latter contain intranuclear "inclusion bodies".

Inclusion bodies have been described in many proven virus diseases. Their true nature is still a matter of dispute and outwith the scope of the present study. Whatever it may be, with a few reservations (Cowdry, 1940), they often represent the morphological stigmata of virus infection.

It is widely believed that Verruca Vulgaris is a virus disease on the basis of clinical studies (McLaughlin & Edington, 1937; Grigg & Wilhelm, 1953) and from the transplantation experiments of Wile and Kingery (1919) and Kingery (1921).

Since the report of Woodruff and Goodpasture (1931) of the cultivation of the fowlpox virus, the developing egg has been used extensively in the investigation of virus disease. References to this technique being utilized in the study of verrucae are, however, scanty. Felsher (1947) and Fischer (1953) record negative results with comparatively small series. Bivins (1953), on the other hand, produced massive lesions of proliferation and "pearl formation" on the chorio-allantois of ten day old chick

embryos with a bacteriologically sterile extract of wart material from his own hand.

It seemed reasonable, therefore, to study the effects of wart material of both types on the CAM and other sites in the developing egg, and to determine (1) whether consistent lesions were producible and (2) whether there was any difference produced by material from the two types of lesion. Human skin was successfully grafted on the chick chorio-allantois by Goodpasture et al. (1938) and subsequently used by them to study fowlpox (1940) and herpes zoster (1944). As no reference to this technique being utilised to study human papillomata could be found, it was decided to inoculate similar grafts of epithelial tissues.

The number of lesions inoculated in the various sites are summarised in table VI (p. 180). No lesions were produced in yolk sac, amnion or brain and negative results were obtained by the subsequent reinoculation of material from these sites on the chorio-allantoic membrane. Skin was the only human tissue which it was found possible to graft with consistent success (Table VII, p. 181). Therefore the remainder of this section is confined to the lesions produced in two sites by cell free, bacteriologically sterile material from both types of wart. These are (1) the chorio-allantoic membrane and (2) human skin grafted on this membrane.

INOCULATION OF THE CHORIO-ALLANTOIC MEMBRANE

METHOD

Each wart was divided into four pieces and one of these was stored at -4°C, within 30 minutes of removal.

Subsequently it was ground with sterile sand and 1 ml. of saline, using a sterile pestle and mortar. The resulting suspension was centrifuged for 15 minutes at 2000 r.p.m. to remove the solid debris. The supernatant fluid in initial experiments was divided into two equal parts. 0.1 ml. of sterile distilled water containing 100 units of penicillin and 100 µgm. of streptomycin was added to one portion, while the other was passed through a Bijou pad filter. This antibiotic solution was found to be adequate for the inhibition of bacterial growth. Microscopic examination confirmed that the resultant fluid was free from cells and cultures were carried out in broth and on blood agar plates to confirm the absence of bacteria. This resulting cell free and bacteriologically sterile fluid was then introduced into the fertile hen's egg.

In general, the methods used were those described by Beveridge and Burnet (1946). The only modification introduced was in the 'dropping' of the chorio-allantoic membrane. After candling and removing a 1 cm. square of shell, a small hole was

made in the air sac and negative pressure applied by means of a rubber teat. No fluid was found necessary subsequently to separate the shell membrane, which was punctured with a dissecting needle. This method was considered to give less non-specific reaction than that using fluid. Ten day embryos, from white Leghorn hens, were mainly used and in all cases three eggs were inoculated with material from one lesion and the tissues removed for examination after 3, 7 and 10 days.

The membranes and other tissues were fixed in 10% neutral formalin and paraffin sections prepared and cut at 6μ. They were stained by the various methods used in the human skin lesions.

At the time of each inoculation, controls were set up using saline of the same batch and similarly prepared extracts of normal skin.

RESULTS

A. Morphology: The chorio-allantoic membrane has been studied in considerable detail and no consistent macroscopic lesions have been found. As illustrated in figure 25 (p. 219), the normal CAM consists of three layers; a central highly vascular mesoderm which is bounded by the endoderm, which consists generally of a single layer of low cuboidal epithelium and superficially by a single layer of considerably flattened epithelial cells - the ectoderm.

In control eggs which have been opened and sealed without the

introduction of an inoculum, there is generally only slight proliferation of the ectoderm. It is now composed of two layers of cuboidal cells. This lesion is patchy and generally confined to the area immediately underneath the opening in the shell. The control membranes, which have been inoculated with extracts of normal human skin, show fairly pronounced lesions. The mesoderm shows moderate oedema and increased vascularity. It is also considerably infiltrated by fibroblasts. The endoderm occasionally shows small patches of cellular proliferation, but the most striking lesions are those in the ectoderm. In general, it is now several layers thick; the cells are larger and have a resemblance to squamous cells, being relatively large, round or oval and becoming flattened as the surface is approached. The nuclei, which are large and central, consist of a delicate network of chromatin in which there are prominent eosinophilic nucleoli. Superficially there is often a thin layer of structureless eosinophilic material. in which there may be an occasional parakeratotic nucleus (fig. 26, p. 220).

The lesions produced on the chorio-allantois by extracts of warts are, by comparison, striking. At the outset it must be stated that there are no consistent morphological differences produced by the extracts from the two types of lesion. Examination of numerous sections again fails to show any evidence of inclusion bodies. In addition, no "pseudo-inclusions" of the type described

by D'Aunoy and Evans (1937) in uninoculated membranes have been demonstrated in any of the preparations. The changes consist of considerable ectodermal proliferation with infiltration of the mesoderm by clumps of hyperplastic ectodermal cells. In places these cells contain a central core of eosinophilic keratin-like material (Fig. 27, p.221). These structures closely resemble the cell nests found in squamous cell carcinoma. The individual cells are large with pale slightly granular cytoplasm, which is eosinophilic. There is a large central vesicular nucleus with a deeply basophilic nuclear membrane and large pale eosinophilic nucleoli. Mitotic figures are moderately frequent. The cells show a distinct resemblance to Malpighian cells but intercellular bridges are not present. Superficially these hyperplastic areas are covered by deeply eosinophilic refractile material which has the morphological features of keratin.

Areas which are indistinguishable on morphological grounds from parakeratosis are also present above this hyperplastic epithelium. Keratohyalin granules are not however present. The mesoderm is markedly thickened and oedematous. There is increased vascularity and widespread infiltration by fibroblasts and small mononuclears. The endoderm also is thickened and consists of about three layers of darkly staining cuboidal cells.

As shown in Table VI, 10 membranes were dissected from eggs, prepared in the same manner as the original wart material, and

each reinoculated into 3 eggs. Of these membranes 7 had been originally inoculated with hyperkeratotic lesions and 3 with dyskeratotic lesions. No differences could be detected between the two groups. The findings both morphologically and histochemically are essentially the same as those found in membranes inoculated with the control extracts of normal human skin.

B. HISTOCHEMISTRY

The histochemistry of the hyperplastic epithelium resembles that of the squamous papillomas, there being relatively little alteration in the DNA content apart from increased density due to nuclear condensation. The demonstrable RNA content appears to be markedly increased in the more actively growing cells but absent from those in the process of mitosis or forming keratin.

Glycogen, as demonstrable by the PAS method, is mainly present in the cuboidal cells of the endoderm (Fig. 28, p.222). As shown in figure 29 (p. 222) however, it becomes a striking feature in the hyperplastic membrane, which has been inoculated with normal skin or in the earlier stages of those inoculated with wart material. It disappears as keratin is formed.

The sulphydryl groups also are slightly reactive throughout the ectodermal portion of the membrane, the mesoderm and the endoderm being unreactive. As the ectodermal layer becomes thicker and more hyperplastic the concentrations of the SH groups become greatly

increased (figure 30, p. 223). Again there is a band of intensified staining at the junction of the ectodermal and keratin layers. A fairly high concentration of sulphydryl is also present in the "keratin layer" (figure 31, p. 223).

The disulphide reaction is almost entirely confined to the apparently fully keratinised zone and in this situation it is intense (figure 32, p. 224). A similar reaction is found in the "keratinised" zone of the "cell nests" in the mesoderm (figure 33, p. 224).

INOCULATION OF HUMAN SKIN GRAFTS ON THE CHORIO-ALLANTOIC MEMBRANE

METHOD

This is essentially a slight modification of that originally described by Goodpasture et al. (1938). Initially considerable difficulty was encountered but eventually the technique now described gave consistently good results with human skin as shown in Table VII (p. 181).

Ten day eggs were generally used, since this was the earliest age at which a consistently high percentage of 'takes' could be obtained. If younger embryos were used, the skin grafts floated for a variable period and then rounded up to form cysts in which the epithelium became necrotic. Deaths of the younger embryos was also a frequent occurrence.

After candling and locating the CAM a window, 1 cm. in diameter, was cut in the shell with a carborundum disc, driven by a portable dental drill. The shell was removed and the membrane dropped in the usual way by creating negative pressure in the air sac. Skin was then placed on the CAM and the opening sealed with cellophane tape. The eggs were then incubated at 35.5°C in a Hearson's egg incubator.

The human skin was obtained from the Plastic Surgery Unit and consisted of excess material which was to be used for grafting

purposes. In general it was taken from the anterior aspect of the thigh or the anterior abdominal wall. It consisted of the whole thickness, i.e., epidermis and part of the underlying upper dermis.

The specimens received were generally 3 x 1 cm. and thicker at one edge than the other. From the thinner portions squares measuring 0.25 x 0.25 cm. were cut with scalpels and placed on the CAM. It had been found by preliminary experiments that with pieces of this size little or no curling occurred at the edges, but anything larger required mechanical flattening and risk of injuring the delicate underlying membrane. Even with the greatest possible delicacy of handling, many of them did not take and within 48 hours they were generally completely necrotic.

In the experiments involving the inoculation of wart material, however, it was found that larger pieces were required. It was found quite possible to graft pieces 1 cm. square by the simple manoeuvre of flattening the skin surface downwards on a thin layer of "tulle gras". This square was then placed on the CAM, resealing the opening with cellophane tape. Four days later the egg was re-opened and the tulle gras removed. In those eggs which survived, and by this time generally 9 out of 10 did so, the skin surface was lightly scarified and 0.1 ml. of wart extract was evenly distributed over the top of the skin. Details of the numbers are given in Table VI (p. 180).

Five days after inoculation the membrane with the attached

grafted skin was removed and fixed intact. Paraffin embedded sections were prepared. The staining methods used were the same as those used in the study of the warts themselves. Control preparations were again inoculated with normal saline and extracts of normal skin.

RESULTS

No macroscopic changes are produced in any of the grafts. In some instances the adjacent membrane shows small areas of thickening and opacity around the graft and occasionally there are tiny areas of haemorrhage. Later, the blood vessels become congested and larger and can be seen entering the graft with the naked-eye (figures 34 and 35, p 225).

The uninoculated grafts of human skin are generally firmly adherent within 24 hours. The ectodermal layer beneath the graft becomes swollen, later degenerates and generally disappears completely by the 5th day. The dermal surface of the grafted skin merges into the mesoderm, where there is some increased vascularity and fibroblastic activity. Young fibrous tissue is formed later. Initially the mesoderm is swollen but becomes considerably thinned after 4-5 days. Occasionally fragments of the ectoderm survive and form nodules of hyperplastic cells which have a distinct squamous appearance and which, in time, develop into partially keratinised cell nests (figure 36, p.226). A few

inflammatory cells are also present and there is an occasional multinucleated giant cell. The endoderm in the region of the graft also shows pronounced hyperplasia. The cells are low cuboidal and reach a maximum thickness of 4-5 layers. Notable changes are also present in the graft. In the dermis there is increased vascularity. The vessels become dilated and contain nucleated red cells proving the continuity of the dermal vessels with those of the chick mesoderm (figure 37, p.227).

The epidermis almost always remains well preserved. The basal cells generally tend to become elongated and less densely staining. Mitotic figures are perhaps slightly more numerous than is usual in normal human skin from the same regions (thigh and abdomen). It gradually becomes thinner, the squamous cells becoming rather more eosinophilic and the nuclei smaller. Mitotic figures are very infrequent. Intercellular bridges are well preserved. There is a moderate increase in the amount of keratin but keratohyalin granules are not a prominent feature (figure 38, p. 228). In brief the appearances are those of normal healthy skin which is continuing to produce keratin. This is borne out by the histochemical findings which again are similar to those of normal healthy skin, except that there appears to be rather less RNA in the lower portion of the Malpighian cell layer and more in the basal cell layer. Glycogen is scanty and, where present, only occurs in isolated cells low in the squamous layer. The

distribution of the SS and SH groups is essentially the same as that of normal skin. The changes in the control grafts inoculated with extracts of normal skin are essentially similar to those which have not been inoculated.

By comparison with both these groups, the changes in the group inoculated with wart material are striking. They are mainly in the epidermis which shows moderate acanthosis and, while there is no frank papillomatosis, there is some extension of the interpapillary processes into the underlying dermis (figure 39, p.229). The basal cells are now definitely cuboidal and show fairly frequent mitotic figures, but the basement membrane is intact throughout. The Malpighian cells show slight pleomorphism and moderate mitotic activity. Intercellular bridges are not so well developed. There is moderate hyperkeratosis and, in places, some parakeratosis. Many of the more superficial Malpighian cells show vacuolation but keratohyalin granules are not a prominent feature; in fact, quite the reverse is the case. No inclusion bodies have been seen and no differences detected between those inoculated with extracts from either type of wart. Eventually the greater part of the squamous layer is keratinised. There are frequent collections of parakeratotic cells and actively growing "basal" cells which are invading the underlying dermis (figure 40, p. 230).

The histochemical findings compared with the control grafts consist of the following. There is a fairly pronounced increase

in the amount of RNA in both the basal and lower squamous layers, which rapidly diminishes as the surface is approached. An increase in the amount of demonstrable glycogen occurs in isolated cells in the lower squamous layer. It appears to be more prominent in those areas which are associated with an overlying parakeratosis.

There is a pronounced increase in the amount of sulphydryl. This occurs in both the squamous and keratin layers. It is particularly intense in the latter site (figure 41, p. 231) where it is considerably greater than is seen in uninoculated skin. This increase is also demonstrable in those areas of hyperplastic epithelium which extend into the dermis and in their central masses of keratin-like material (figure 42, p. 231). The reaction for disulphide is confined to the keratin layers. It is faint and stains only to an estimated concentration of 25% of that of normal skin.

DISCUSSION

The inoculation of the chorio-allantoic membrane of the chick is now a standard technique in virology. Its possibilities were probably first demonstrated by accident by Rous and Murphy in 1911, who noted that, in eggs inoculated with filtrates of the Rous chicken sarcoma, tumour developed on this membrane.

The greatest impetus, however, stems from the work of Goodpasture and his colleagues (Woodruff & Goodpasture, 1931; Goodpasture et al., 1932). They showed that inoculation with material from fowlpox resulted in the development of characteristic inclusion bodies in the cytoplasm of the affected cells (Bollinger Bodies). Similarly, vaccina could be carried in serial passage with the development of Guarnieri bodies. Some of the viruses which have been cultivated by this method produce sufficiently distinctive gross appearances to permit diagnosis on naked-eye examination, e.g., fowlpox, vaccinia and infectious laryngotracheitis of fowls.

These appearances are admirably summarised by Beveridge and Burnet (1953). They point out, however, that this membrane histologically is of almost diagramatic simplicity and, therefore, it is only to be expected that the lesions produced on it will have many features in common, and the appearances produced will depend on the relative degrees of proliferation and necrosis.

The chorio-allantoic membrane, is, of course, a site which is

notorious for non-specific lesions. In addition to the undoubted changes produced by viruses, it has been shown by Goldsworthy and Moppett (1935) that localised lesions can be produced by experimental evaporation of the membrane. These show little change in the ectodermal layer, slight mesodermal oedema and intense endodermal hyperplasia. There is vacuolation of the endodermal cells in which inclusion bodies occur. Some of these are intranuclear, but for the most part they are not in relation to recognizable intact nuclei. D'Aunoy and Evans (1937) described various histological changes in the chorio-allantois of unopened eggs. These consist of varying degrees of mesodermal oedema and ectodermal and endodermal proliferation. Where ectodermal proliferation is pronounced many of the cells are vacuolated and contain rounded bodies which vary in size and which are unrelated to nuclear chromatin.

In the present series no macroscopic lesion has been demonstrated on the membrane and no inclusion bodies found. Thus no difference in behaviour can be detected between the two main types of lesion. It must be concluded that, under the conditions of these experiments, the chorio-allantois does not support the growth of the virus of verruca, which on previous clinical and experimental evidence does appear to exist. These findings do not confirm those of Bivins (1953), and suggest that the agent isolated by him was not that of verruca vulgaris and probably a contaminant. Although no firm conclusions can be reached possible explanations can be considered.

Thus it may be that using hand pestle and mortar failed to cause sufficient disintegration of the cells to release the virus. This is unlikely as subsequent experiments were undertaken and microscopical examination revealed destruction of the cells, although a moderate number of nuclei were still intact. It is, of course, well established that many viruses will not grow on the membrane although they can be cultivated in tissue cultures. Poliomyelitis is the outstanding example. In the case of warts, an additional factor may be connected with the long incubation periods which have been reported (Templeton, 1935; Lyell & Miles, 1951).

What is possibly more relevant to the present study is the gross hyperplasia of the ectoderm with the production of material which has the morphological and most of the histochemical characteristics of keratin. In view of the failure to produce any consistent macroscopic lesions, the lack of difference in behaviour in two apparently distinct lesions, and most important of all the fact that these changes could not be transmitted to a second generation of eggs render the possibility that these lesions are the result of virus activity purely speculative. It remains, however, that inoculation of the membrane with cell free, bacteriologically sterile, filtrates of human wart material produces gross hyperplasia. This is consistently much more pronounced than in lesions produced by the inoculation, under identical conditions, of the same amount of "control" material, such as saline extracts of normal

skin.

Beveridge and Burnet (1953) discuss the possible mechanism of ectodermal proliferation in membranes inoculated with virus. They suggest that after a cell is damaged as the result of virus activity within it, the neighbouring ectodermal cells proliferate in response to something diffusing from the damaged cell. This may be either virus particles or as they consider more probable, growth stimulating substances resulting from the primary damage. This may be related to the hypothetical wound hormone which was at one time so popular. Whatever the nature of this substance, which is outwith the scope of the present discussion, there appears little doubt that damaged cells alone are sufficient to provoke hyperplasia of the ectoderm. Extracts of hyperplastic lesions, such as warts, compared with normal skin consist of material from much more rapidly growing and, therefore, probably much more rapidly degenerating cells. Consequently it is not unreasonable that these lesions should produce considerably more hyperplasia of the ectoderm. This may be supported by the findings of Meirowsky et al. (1954). They inoculated the chorio-allantois with Seitz filtered material from two cases of intraepidermal carcinoma. The lesions on the membrane which they describe and illustrate are very similar to those seen in the present study, although these authors claim that they are specific.

As might be suggested by its highly vascular nature, this

membrane is primarily a respiratory organ (Romanoff, 1952). The proliferation of the ectoderm takes place with remarkable rapidity, and the most striking feature is the similarity of these hyperplastic cells to those of squamous epithelium. Although intercellular bridges have not been seen, the resemblance is otherwise complete. The resemblance again of the cellular collections in the mesoderm to cell nests is remarkable. Metaplasia to a squamous type of epithelium is, of course, a frequent finding in various reactive and neoplastic processes in human tissues. It often proceeds to the formation of keratin. On morphological grounds there would appear to be keratin formation both on the surface of the hyperplastic ectoderm and in the centre of the "cell nests". Although these are generally accepted as an indication of a fairly high degree of differentiation in a squamous cell carcinoma, they nevertheless are present in what is a rapidly growing lesion. Thus the process of keratinisation as it occurs in the ectoderm is more rapid than that which normally occurs in human skin or in the hyperplastic lesions as illustrated by the two types of warts.

The histochemical findings are essentially in agreement with this suggestion. Thus there is increase in the amount of demonstrable glycogen in the hyperplastic cells and this decreases with the onset of keratinisation. The sulphydryl groups also increase with the assumption of the morphology of squamous cells. This gradually becomes more intense and the zone of transition or

the keratogenous zone, as demarcated by a zone of intensified staining for sulphydryl, has become much broader.

Again, although the disulphide reaction is confined to the keratinised areas of the ectoderm and the cell nests, in both of these sites, there is still a high concentration of sulphydryl and, although there is some reduction as compared with the squamoid zone, this is even less marked than is seen in the hyperplasias which, it may be recalled, are again less than that found in normal keratinising human skin.

Thus it seems reasonable to conclude that although there is no evidence to point to its identity, much less to suggest that it is a virus, cell free filtrates of human skin lesions can provoke the proliferation of the chick ectoderm which, although it may be an exaggeration of a non-specific reaction, is characterized by the formation of cells which become squamous in type and which form material which is indistinguishable from keratin on morphological and histochemical grounds.

In addition to the voluminous studies which have been made in respect to the effects of viruses on the chick chorio-allantois, an extensive literature has grown up describing the transplantation of various human and animal tissues on the membrane. Much of the work has naturally been concerned with the transmission of tumours of various kinds (Campbell, 1949). Again skin has been a relatively neglected field despite the excellent original studies of Goodpasture

et al. (1938, 1944). This group again showed that successful transplants could be made and that they could be utilised in virus studies. There are, in addition, records in which adult human skin could not be transplanted although benign and malignant tumours could be transplanted readily (Mirand and Hoffman, 1953).

After some initial difficulty with the technique it was found possible to successfully explant skin with consistently successful results. Squares up to a diameter of 1 cm. took readily. On reflection, this is not as surprising as it seems. Oakley (1938), who transplanted liver on to the membrane, said "there seems to be a prejudice in favour of small transplants - if vascularisation is excited by the products of autolysis a larger transplant stands a better chance of vascularisation than a small one". The vascularisation of the graft can be shown by the presence of nucleated red cells in the capillaries of the human dermis, and there appears little doubt that they remain in a healthy condition once they have "taken". There is relatively little in the way of reaction on the part of the membrane to the graft for, as already mentioned, the ectoderm underneath it has generally disappeared by the fifth day but, where it survives, "the cell nest" like structures of essentially similar appearance to those described above develop. In the controls the appearances are essentially those of normal skin which is continuing to produce keratin. There are also no significant differences in the histochemical findings.

Again, the skin grafts inoculated with cell free and bacteriologically sterile filtrates show consistent and distinctive changes when compared with the control preparations. There is, however, no demonstrable difference in the effects produced by extracts from the two types of lesion. It may be reasonable to assume however, that non-specific reactions would be less likely to occur in this situation than on the membrane itself. Whatever may be the cause, these grafts show hyperplasia of the squamous cell layers and of more direct interest an increase in the amount of keratin formed. There is, however, no papillomatosis and one can only speculate whether, if the time of incubation could be prolonged, this would develop.

Associated with this increase in the rate and amount of keratin formed there are histochemical differences. Thus, once again, the inverse relationship between the amount of demonstrable glycogen and the degree of keratinisation can be demonstrated. The distribution of sulphydryl again is similar to that found in the sites of more rapid keratinisation in that the reduction in amount in the keratin layer is less pronounced than in normal controls. The concentration of disulphide in the keratin layer is also much lower. Thus, the findings are in keeping with those in the hyperkeratotic lesions which are assumed to be growing more rapidly and in which the process of keratinisation is also more rapid and consequently possibly less complete.

Moreover, in these grafts the accelerated rate of growth can be proved when they are compared with the controls, both specimens being originally from adjacent portions of skin removed from the same patient. Therefore, whatever the cause, there is considerable increase in keratin which has been formed more rapidly. The histochemical findings are also consistent with it not being completely formed.

SUMMARY

Cell free and bacteriologically sterile extracts of warts of the hyperkeratotic and dyskeratotic types have been inoculated on to the chorio-allantoic membrane of the hen's egg. No morphological differences can be demonstrated between the lesions produced by either type, and no inclusion bodies develop. Both types, however, produce gross hyperplasia of the ectoderm which becomes squamous in type and produces keratin. Hyperplastic epithelium extends deep into the mesoderm forming structures closely resembling cell nests. Again these contain keratin.

Human skin has been grafted on to the chorio-allantoic membrane and inoculated with extracts of both types of warts. Again no differences in reaction can be demonstrated between them, and no inclusion bodies develop. Hyperplasia of the squamous cell layer is produced with considerable increase in the amount of keratin formed.

In both sites the process of keratinisation is associated with a decrease in the amount of demonstrable glycogen. Sulphydryl also diminishes with the formation of keratin but to a considerably lesser extent than that in normal keratinising human skin or in the hyperplastic lesions of both types. The concentration of disulphide in the keratin layer is also considerably lower. It is suggested that these findings represent an incomplete form of

keratinisation as the result of the accelerated rate of formation.

PART IV

VAGINAL KERATINISATION IN MICE BEARING HORMONE SECRETING TUMOURS

INTRODUCTION

A different experimental approach to the present study was afforded during a year spent in the laboratory of Dr. Jacob Furth. He and his associates developed transplantable tumours from mouse pituitary glands. These tumours secrete pituitary hormones and produce widespread effects in the host animals. Two such tumours were studied in detail in Boston and are now carried in LAF 1 mice in Glasgow (the technical details are given in the appendix, p. 293). In animals bearing a tumour which secretes corticotrophin there is abolition of the periodic keratinisation of vaginal epithelium. The other tumour secretes thyrotrophin and in these animals there is evidence of marked gonadal stimulation. This section is concerned with the hormone dependent keratinisation of mouse vaginal epithelium which occurs during oestrus. Accordingly, after a brief consideration of the more general effects produced by these tumours, the morphology and histochemistry of vaginal keratinisation in normal LAF 1 mice will be described. Then follows an account of the effects produced on this process by these tumours and their subsequent modification under experimental conditions.

A. ADRENOTROPHIC PITUITARY TUMOURS (ADRENOTROPES)

These were the first pituitary tumours to appear after total body irradiation and several transplantable strains were developed (Furth, 1955). These tumours proved, unlike the thyrotrophic tumours to be autonomous in the first transplanted generation in that they could be grown in normal hosts.

The findings in animals bearing such tumours have been well described (Bahn et al., 1957) and will be given here only in outline. The characteristic gross anatomical features include enlargement of the adrenal glands (figure 43, p. 232), involution of the thymus and obesity (figure 44, p. 233). Lymphopenia, eosinopenia and polyuria are present before the tumour is palpable. The latter grows rapidly after a variable latent period which gradually becomes less with each subsequent transplant (figure 45, p. 234). The animal generally dies before the tumour reaches a diameter of 1 cm. A further constant effect is increased sensitivity to infection, the animal usually dying of endogenous infection by saprophytes, especially diphtheroids. The histological appearances of the tumour are shown in figures 46 and 47 (pp.235, 236). Of particular interest to the present study is the finding that vaginal keratinisation is often irregular. All the changes produced by this tumour can be prevented by adrenalectomy which also enhances tumour growth.

It has been shown that in the intact animal with an adrenotrophic tumour there are high blood levels of corticosterone and 11β hydroxy-Δ_4-androstene 3.17 dione; 11α hydroxyandrostane-3.17 dione has been identified in the urine (Wilson et al., 1958).

Cohen and Furth (1959) using an in vitro assay method with adrenal tumour slices as a source of steroid found 4-55 milliunits/mg. of corticotrophin in sucrose homogenates of these tumours. Steelman et al. (1956) previously found lower levels with an in vivo method. The strains of tumour used, however, were older.

B. THYROTROPHIC PITUITARY TUMOURS (THYROTROPES)

Thyrotrophin secreting tumours rarely occur in LAF 1 mice irradiated with X-rays and then only in about the same percentage as in unirradiated controls (Furth et al., 1959). Tumours, however, can be produced readily by complete or nearly complete destruction of the thyroid by I^{131} (Gorbman, 1949). Surgical thyroidectomy, although seldom complete, can also produce such tumours (Furth, 1957). Similar tumours have also been produced in mice by the administration of a thiouracil derivative (Moore et al., 1953).

Unlike the adrenotropes the thyrotrophic tumours are initially dependent in that they will grow only in animals whose thyroid function is deficient (Furth et al., 1953). After several serial transplants, however, autonomous variants generally arise and

can subsequently be carried in intact hosts.

The level of circulating hormone in animals with still dependent thyrotrophic tumours is enormous, reaching some 2,000 times that of the usual blood levels. Tumour tissue has a concentration of 40 U.S.P. units/g. on a wet weight basis compared with an estimated 20 U.S.P. units for the normal mouse pituitary.

Autonomous tumours have shown lower but still grossly elevated levels of thyrotrophin. Thus, in the strains studied personally, which were in the fourth passage, the levels in the tumour ranged from 12-15 U.S.P. units/g. wet weight (Kamat, 1959).

Autonomous thyrotrophic tumours can cause widespread hyperplasia of the host's thyroid. The histological changes range from initial slight papillary infolding to widespread solid areas which show many of the histological features associated with malignancy (figure 48, p. 237). These lesions have been shown to be transplantable but, so far, only to animals which also have a thyrotrophin secreting tumour and therefore a high blood level of thyrotrophin (Ghera, et al., 1960). Thus they themselves are still dependent neoplasms.

Of the secondary changes which are present in these animals, of particular interest is the gonadotrophic 'side effect', which is present in mice bearing highly functional thyrotrophic tumour (Furth & Clifton, 1957). In such animals the ovaries are increased

greatly in size and often contain multiple cysts. The uterine horns are also markedly dilated (figures 49 and 50, pp. 238, 239).

KERATINISATION OF THE VAGINAL EPITHELIUM DURING THE NORMAL OESTRUS CYCLE

A. MORPHOLOGY

The cycle was followed by vaginal smears. Details of the technique are given in the appendix (p. 293). It is well known that in some strains of mice, if daily examinations only are made, pro-oestrus and the first part of oestrus period may not be detected. In the initial studies, therefore, smears were made at 12 hourly intervals. Thereafter 20 normal LAF 1 mice were examined daily at the same time each day. The animals were killed at various stages in the cycle and histological preparations made. The following cycle was found to be regularly present throughout.

Di-oestrus is the resting or quiescent stage and in these animals lasts 2 days. The epithelium consists of 4-5 layers of rather flattened squamous cells - mitotic figures are very rare and keratin is not seen. Vaginal smears at this stage consist of large nucleated epithelial cells and a moderate number of polymorphonuclear leucocytes.

Pro-oestrus: This generally lasts only 1 day. The epithelium is now 8-10 layers in thickness. The outermost cells are still nucleated and amongst them there are leucocytes. Beneath them are keratinising cells in which keratohyalin granules are present. These granules, however, are very small and never at any stage a prominent feature. The lowermost layers of cells are typically

squamous in appearance and the basal cell layer contains a moderate number of mitotic figures. The vaginal smear at this stage consists of lightly staining nucleated cells.

Oestrus: This generally lasts 2 days in these animals. There is superficially a dense layer of keratin and underneath 8-12 layers of squamous cells. Keratohyalin granules are no longer prominent and mitotic figures are infrequent. The smear now consists solely of adherent flakes of keratin.

Meta-oestrus: This phase lasts 1 or occasionally 2 days. The superficial keratin layer sloughs off together with part of the squamous layer. The remaining part of the squamous layer is heavily infiltrated by leucocytes. The smear at this stage consists of groups of keratinised cells which are surrounded by large numbers of polymorphs.

B. HISTOCHEMISTRY

Again, of the histochemical stains employed, only two were found to be of any real value - the periodic acid-Schiff for glycogen and the dihydroxy-dinapthyl-disulphide reaction for the sulphydryl and disulphide groups.

Early in di-oestrus there is a faintly positive sulphydryl reaction in the cytoplasm of the epithelial cells, the nuclei being unreactive. This reaction is initially uniformly distributed throughout the epithelial layer (figure 51, p.240), but later

more intense staining is present in the lower half of this layer. No reaction can be detected in the superficial swollen nucleated cells. All layers at this stage are completely unreactive for disulphide. There is a moderately intense reaction for glycogen. This is present as prominent granules in the cytoplasm of the upper 2 or 3 layers of the squamous cells.

In pro-oestrus the lower 6 or so layers of the epithelial layers again show slight reaction for sulphydryl, while the superficial cells are unreactive. The intermediate zone of partial keratinisation shows a much higher concentration of sulphydryl and a faintly positive reaction for disulphide. The reaction for glycogen is still moderately intense and it is largely confined to the lower and middle parts of the squamous layer. The outermost prekeratinised layers are almost completely unreactive.

At oestrus the epithelial layer shows moderate staining for sulphydryl groups throughout and an intensely staining band is present at the junction of the squamous and keratin layers. This includes the S. granulosum and extends well into the keratin layer (figure 52, p. 240). The remainder of the keratin layer is also moderately positive for sulphydryl but intensely so for disulphide (figure 53, p. 240). The reaction for glycogen at this stage is least intense and confined to the middle zone of the squamous layer, the superficial keratinising layers being unreactive.

In meta-oestrus the sulphydryl reaction in the remaining

squamous layers is now considerably less intense and there is no reaction for disulphide. The reaction for glycogen is, on the other hand, stronger and present in what is now the upper half of the squamous layer.

THE EPITHELIUM IN MICE BEARING ADRENOTROPHIC TUMOURS

Two strains of tumour were studied, Nos. 20 and 2. The latter is thought to have higher levels of circulating hormone. Possible differences between them will be discussed further later. The sequence of transplantation and their latency are given in Tables VIII and IX (pp.182,183). The operative treatment of the animals of strain 20 whose oestrus cycles have been followed is also given in Table VIII (p. 182) and summarized in Table X (p. 184). In strain 2 it was only possible to follow intact animals.

The results are summarized in Tables XI and XII (pp.185,186). The cycle as shown by the control animals, i.e., intact animals without tumour, is regular and as given previously. The animals bearing an adrenotrophic tumour show gross alteration in the cycle. Thus in strain 2 (Table XII, p. 186) the last detectable oestrus phase was 14 days prior to the tumour becoming palpable and thereafter the animals give smears which are typically those of the pro-oestrus phase. There are no cornified cells. Sections of these vaginas show that the epithelium consists of 2-3 cell layers in thickness. There is an inner zone consisting of a single layer of small cuboidal cells with relatively large central nuclei and deeply eosinophilic granular cytoplasm. These cells contain abundant ribonucleic acid. Glycogen is virtually absent and they

are completely unreactive for sulphydryl or disulphide. Superficially there is in most places a single layer (occasionally it is double) of tall columnar cells with small darkly staining nuclei which are basal in position. The cytoplasm which is faintly eosinophilic contains abundant Schiff positive material, which may be mucin. There is no reaction for ribonucleic acid, sulphydryl or disulphide groups, and no evidence of keratin formation (figure 54, p. 241).

As will be seen in Table XI (p. 185) the cycle in the other group of tumour bearing animals (strain 20) is also considerably altered. Thus, for the greater part of the cycle the smears are indistinguishable from those of late di-oestrus or early pro-oestrus in the intact normal animal. At infrequent intervals the animals go through a very brief - always less than 12 hours - phase which has been designated P/O. The findings in the smear are predominantly those of pro-oestrus. There are mainly lightly staining but still nucleated cells and occasionally a few completely cornified cells. Sections of vagina taken at this stage are as shown in figure 55 (p. 241). It consists of some 6-7 cell layers. In the lower layers the cells are still oval and have nuclei which now almost fill the whole of the cell and which stain lightly. These cells have a moderate content of ribonucleic acid but are unreactive for glycogen, sulphydryl or disulphide. Superficially there are two layers of flattened cells. In the

lower the nucleus, which is now also flattened, stains fairly densely and the cytoplasm is finely granular. In the outermost layer the nuclei are elongated and stain very densely. The cytoplasm is now more deeply eosinophilic. There is no morphological evidence of keratinisation and keratohyalin granules have not been identified. A moderate number of polymorphs are present throughout this epithelial layer.

Oestrus Cycle in Adrenalectomised Mice Bearing Adrenotrophic Tumours

The number of adrenalectomised mice bearing tumours studied is given in Table X, (p.184). The sequence of their oestrus cycle is also given in Table XI, (p.185). The only detectable difference between these and the intact non-tumour bearing controls is that the meta-oestrus stage is prolonged and the di-oestrus stage is shortened. The duration and frequency of the oestrus stage is, however, unchanged. Sections examined from the vaginas of these animals killed at various stages of the cycle reveal no notable morphological or histochemical features between the two groups.

Gonadectomised and Adrenalectomised Mice Bearing Adrenotrophic Tumours

The number of animals studied is again given in Table X and the findings are summarised in Table XI.

It will be seen from this table that there is no evidence of keratinisation as shown by the vaginal smears, which are extremely scanty in cell content. The animals are almost continuously in a state of pro-oestrus with occasionally phases when the appearances are those of di-oestrus. Histologically, the vaginal epithelium consists of a double layer of cuboidal cells which have relatively large central nuclei which stain densely (figure 56, p. 241). The cytoplasm is pale and eosinophilic. There is a slight reaction for ribonucleic acid. Glycogen is only present very infrequently as sparsely arranged fine granules. There is no reaction for sulphydryl or disulphide. There is no evidence of keratinisation and no structures resembling keratohyalin granules are seen.

Oestrus Cycle in Thyrotrophin Tumour-Bearing Mice

The number of animals studied is given in Table XIII, (p. 187). They are in two groups, intact and athyroid. This latter group received 60 μc. I^{131} intraperitoneally after 10 days on a commercially prepared low iodine diet (Nutritional Biochemical Corp.)

The cycle as assessed by both smears and histological and histochemical examination at various stages is essentially within normal limits for these animals. No difference is detectable between the intact or athyroid groups.

DISCUSSION

This section is concerned with the behaviour of the vaginal epithelium in LAF 1 mice which bear transplantable hormone secreting tumours. They are of pituitary origin and from the original tumours there have been developed readily transplantable strains. Two types have been utilised in this investigation; one which secretes corticotrophin, and the other which secretes thyrotrophin and appears to have a gonadotrophic side effect.

As has been illustrated the morphology of these tumours is not distinctive. In general, histochemical studies have placed them amongst the chromophobes. It is, however, not surprising that they cannot be positively identified on morphological and histochemical grounds with their possible parent cells in the normal pituitary. This probably is of little significance when one recognises that they show several of the criteria associated with malignancy, including cellular pleomorphism. In addition, there may be over-emphasis placed on the separation of the individual cells of the pituitary on the basis of specific staining of their granules. It is conceivable as suggested by Lazarow (1955), that a cell could be synthesising large amounts of hormone and pouring it into the circulation without actually converting it to the storage form which is represented by specific staining granules. This would appear to be quite possible in a

tumour which has a higher rate of cell metabolism than normal.

As a generalisation, as tumours grow more rapidly they tend to become dedifferentiated and to become less highly functional. It has been shown that these hormone secreting tumours which are initially dependent, as in the case of the thyrotrophic lesions have a level of hormone which is higher than tumours which have gained autonomy. Further, with successive transplants the tumours grow more rapidly and gradually the level of hormone secreted declines. This increase in growth rate with accompanying decline in the amount of secreted hormone is also found in the adreno-atrophic tumours although these have been found to be autonomous from their initial isolation. Thus in the two main groups under observation, there are those secreting thyrotrophin and those which secrete corticotrophin. The latter group can again be sub-divided into two groups. There is strain 2, of more recent isolation, which at the time of examination is in the 12 and 13 passages with a mean latency of 96 and 80 days. On the other hand, the animals of strain 20 bear tumours which are now in the 21-29 passages and in which the latency is now reduced to about 30 days. Although it has not been possible toassay directly the corticotrophin level of group 2, this strain should have a higher level of circulating hormone. As will be discussed later, this postulate is in keeping with the findings in the changes in the vaginal epithelium.

The cyclical process of keratinisation in mice since the original work of Allen (1922: 1924) has been extensively studied, and is now the basis of a standard method for bioassay of oestrogens in gonadectomised female mice. It has been shown that, although it is generally present in healthy mice, the regularity and duration of the cycle vary with different strains. In hybrids it has also been found that the vaginal sensitivity to oestrogens is usually intermediate between the two parental strains (Gardner, 1959). In the present study these first generation hybrid mice (LAF 1) do have a regular cycle with an oestrus phase which lasts for two days, as measured by the vaginal smear technique, and subsequently confirmed on numerous occasions by the examination of histological sections.

The examination of the vaginal epithelium at various stages of the cycle has shown that the process of keratinisation is a rapid one and that essentially there is no difference in the morphology or histochemistry of the process as compared with that in normal skin. The process involves the formation of a dense layer of keratin which is completely shed and reformed every 6 days. It shows the same inverse relationship between the amount of demonstrable glycogen and the degree of keratinisation. There is an accumulation of sulphydryl in the keratinising squamous cells, and subsequent reduction in the keratinised layers. Disulphide, again, is only demonstrable in the fully keratinised layers. The

amount of reduction in the keratinised layer compared with the squamous zone is not so pronounced as it is in the normal skin or in the hyperplastic skin lesions already described. This broadening of the transition zone is comparable to that which has been already described in the processes which show evidence of a more rapid formation of keratin, i.e., the hyperkeratotic lesions in human skin or the experimentally produced keratinising lesions described in the previous section.

As mentioned earlier, it is well established that the removal of the ovaries abolishes this cyclical keratinisation and the return of this process can be used as an index of oestrogenic activity of various substances. Biggers and Claringbold (1954) made a statistical analysis of this process on the basis of mitotic counts. They state that it is truly quantal, i.e., the animals either respond or they do not and that no further grading system, of which many types have been suggested, is necessary. They and other members of their group have from time to time suggested that the action of oestrogens in producing keratinisation is through the stimulation of cell division without having any other effect on the keratinising cells.

The rates of mitosis in squamous epithelium, including the vagina, have been studied in considerable detail by W.S. Bullough (1952). He suggests that the most critical nutritional requirement of a cell about to enter division is glucose and this

is related to the high energy requirements which in turn are related to the effects of a wide variety of hormones (Bullough, 1955). He also stated earlier, that the normal action of the oestrogens is related to their capacity to stimulate mitosis throughout the body. Their more vigorous and specialised action on the reproductive organs of vertebrates has probably evolved from this general capacity. He further suggests that the degree of reaction depends on the need for each tissue for cell replacement rather than on the degree of their differentiation (Bullough, 1946).

The precise mechanism of action of the oestrogens remains, however, unproven. The problem is admirably discussed by Mueller (1957) who concludes "at this time we do not know for certain the mode of action of any steroid". More recently Villee (1959) and Rosa and Velardo (1959) have presented chemical and histochemical evidence that in oestrogen sensitive tissues oestrogen acts as a cofactor for isocitric transhydrogenase, thereby increasing the rate of conversion of ATP to ADP thus releasing more energy for metabolic activity. However, it seems at present to be generally acceptable that the production of keratin in the mouse vagina by oestrogens results from the stimulus to mitosis in a site sensitive to the action of oestrogens.

An important part of the last statement is "in a site

sensitive to oestrogens". Thus, while it has been shown by H.F. Bullough (1942-44) that in mice there is a relationship between the oestrus cycle and mitotic activity in skin, the most striking changes are, of course, those in the vagina. However, in addition to there being different sensitivities to oestrogens between different organs, apparently homologous organs react differently in different species. This is illustrated by the reaction of the vagina of the rabbit to oestrogens. It is converted from a simple tube of cylindrical epithelium to one lined by "mucified" cells and in which there is no evidence of keratinisation. According to Jost (1939; 1943), this peculiarity depends on the development of the rabbit vagina from Mullerian elements instead of the urogenital sinus as it is in rats and mice. These observations are supported by Zuckerman (1940). He agrees that oestrogens stimulate not only the growth of the male and female reproductive tissue but other tissues such as skin. He states, however, that generally where the Mullerian ducts contribute to the development of the upper part of the vagina, they are as a rule replaced by sinus epithelium.

As Burrows (1949) has pointed out, this capacity to respond to oestrogen and the degree of responsiveness are innate cellular characteristics. This capacity is retained even when the tissues are transferred to other parts of the body. Thus it has been shown by Raynaud (1930) that the vaginal epithelium of gonadectomised

guinea-pigs, when transplanted to the pectoral region, could respond to the influence of oestrogens by keratinisation.

In the thyrotrophin secreting animals it will be recalled there is no evidence of upset in either the duration or the periodicity of the oestrus cycle. This group may be considered as controls for the corticotrophin secreting animals inasmuch as the presence of highly functional tumours is in itself not sufficient to upset the normal rhythm of the oestrus cycle. A point of more direct interest is that these animals show evidence of gonadal stimulation as evidenced by enlarged ovaries and dilated uterine horns. In the original observations Furth (1955) stated that this only occurred in the athyroid group. In the animals studied personally, however, it is noted that even the intact animals which bear tumours do show some gonadal stimulation. The stimulation, however, is not accompanied by any evidence of alteration in the oestrus cycle, that is the increase in the size of the ovaries and uterine horns is not accompanied by a significant increase in the level of circulating oestrogen.

The changes in the adrenotrophic group are by comparison striking. As was discussed earlier, the oestrus cycle has been followed in two groups; strain 2 of more recent isolation and in which the amount of circulating hormone is probably higher than that in the older strain. This suggestion is supported by the findings in the oestrus cycle. Thus in the strain 2 animals there

is complete abolition of the process of keratinisation and by the vaginal smears the animals are in a continuous stage of pro-oestrus. As will be recalled, in the animals of strain 20, on the other hand, there is occasional and very transient evidence of some keratinisation. This would suggest that the anti-keratinising action of these tumours is more pronounced in the more recently isolated strain than in the older group which is growing more rapidly and in which the level of hormone output is presumably lower.

It is significant that adrenalectomy consistently abolishes this inhibition of keratinisation. There is complete restoration of the normal cycle and within 7 days the smears and histological and histochemical appearances of the vaginas are indistinguishable from the intact normal animal. In the gonadectomised animals there is of course complete abolition of the keratinisation process and the epithelium, as has been illustrated, consists of two layers of cuboidal cells. The appearances of animals which have been both adrenalectomised and gonadectomised are identical.

Thus it appears that in these animals grafted with adreno-trophic tumours, the adrenals are hyperstimulated by the excessive amount of corticotrophin derived from the tumour cells. The adrenals of many species are known to secrete gonadal hormones in addition to the corticosteroids. There is also known to be some similarity of action to the gonadal hormones, particularly between

progesterone and the corticosteroids (Burrows, 1949). Again progesterone has the same fundamental structure as the androgens.

Earlier reports on the effects of corticosteroids on vaginal keratinisation were conflicting. Thus Salmon (1939) found that deoxycorticosterone acetate produced keratinisation in post-menopausal women. Robson (1939), on the other hand, found in mice that deoxycorticosterone inhibited oestrus and caused mucification. The position was clarified by Courrier (1942), who showed that in castrated rats keratinisation of the vagina under the influence of deoxycorticosterone was an early but transitory effect which is soon followed by the so-called "mucification". The action of corticotrophin has also been shown to produce vaginal mucification (Alloiteau & Courvoisier, 1953; Tyan et al., 1953; Schiller, et al., 1953). From the work of the latter there is good evidence that as corticosteroids can completely inhibit the natural and experimental oestrus cycle, there is a double inhibition of both corticotrophin and gonadotrophin in the pituitary. This would appear to be the most reasonable explanation of what happens in the present series. It has been well established in these animals that tumours stimulate the production of large amounts of corticosterone which is the normal steroid of the mouse and as a minor product 11β hydroxyΔ4 androstene 3:17 dione. It is presumably these steroids which, by a

"feed back" mechanism via the pituitary, inhibit the output of oestrogens. Experiments could be designed using hypophysectomised mice on the interaction of the various factors in this hormonal control of keratinisation. It was, however, found impossible to subject hypophysectomised mice which bore these tumours to further operative trauma. Therefore, a more direct approach is made in the next section when the effect of various steroids on keratinisation in tissue culture is considered. The whole question of antagonism of steroids is extremely complex. It may be defined as the suppression or modification of the effect of one hormone on the end organ or tissue by the "opposite" sex hormone. The process has been demonstrated in numerous animal experiments (Burrows, 1949; Roberts & Szego, 1953) but, by and large, it seldom is clear to what extent the antagonism occurs at the site of action on the end organ or is mediated by the pituitary.

Thus, whatever the site of action may be, it has been shown that in these animals the process of keratinisation has been abolished by substances, presumably steroids, which have been produced by the adrenals under the stimulus of a greatly increased output of corticotrophin. These substances are, therefore, antagonists to the normal action of oestrogens.

The phenomenon of "antagonism" will be considered again in the following section. Briefly, however, it has been shown that

vaginal keratinisation can be suppressed by androgens in the intact animal (Robson, 1936), in oestrogen treated hypophysectomised animals (Koch et al., 1950) and in oestrogen treated non-hypophysectomised castrated animals (Emmens & Bradshaw, 1939). It has also been shown that progesterone opposes the proliferative and keratinising action of oestrogens in the vagina (Robson, 1950).

It is obvious that in many reports describing the vaginal changes which are produced by various substances that there is often some confusion as to what precise histological features are involved. Thus the use of the term 'mucifying', which is used to describe the changes which are produced in the vaginal epithelium as the result of the administration of progesterone or as is seen in the adrenotrophic tumour bearing animals, is misleading. The appearance of the cells has already been described and illustrated, but it will be seen that they are not in fact mucus secreting cells as these are generally understood. They are cells which show no evidence of keratinisation and which are being shed complete with nucleus. They represent cells which have not undergone the complete devitalisation as occurs in the process of keratin formation. They have not, however, undergone a form of metaplasia as the term might seem to imply.

Burrows (1949) pointed out that stratification of the vagina, i.e., increase in number of cell layers, precedes the deposition of keratin and appears to be an essential response of the vaginal

epithelium to oestrogen. Thus as Freud (1938) suggests, oestrogens may be classified as 'mitogenic', i.e., they produce a multilayered epithelium. He further classifies testosterone and other androgens as "mitogenic and mucifying", as they produced a multilayered epithelium but with 'mucification' of the superficial layers. He places progesterone amongst the non-mitogenic and mucifying in that there is no great increase in the thickness of the epithelium. It is however mucified only in the sense that it does not show any evidence of keratinisation. Thus, as was discussed earlier, it appears that the primary action of oestrogen is to stimulate the growth of vaginal epithelium and thereby increase the amount of keratin being formed. The action of various substances which may be termed oestrogen antagonists, including the corticotrophin secreting tumours, act through this process of growth. Their effect probably represents a slowing up process suggesting that keratinisation is the common end result of some process of stimulation. This may be chemical as in the case of oestrogens but there is good evidence that keratinisation of vaginal epithelium may be the result of physical stimulation. Thus Wade and Doisy (1935) have shown that in gonadectomised rats, swabbing three times daily was sufficient to provoke keratinisation. It may be suggested that there may be sufficient oestrogens from the adrenals to provoke the keratinisation process but, even if this is so, the physical trauma slight though it may be is an essential

part of the process. Thus keratinisation in the vagina is on histological and histochemical appearances no different from that in any of the other sites which have been described earlier. The available evidence suggests that keratin formation in this site is the end result in considerably stimulated vaginal epithelium and that the inhibition of this process results from the inhibition of cellular proliferation and not through any direct modification of the vaginal cells.

SUMMARY

The oestrus cycle with its periodic vaginal keratinisation has been followed in LAF 1 mice. There is in these animals a regular cycle in which the stage of keratinisation lasts two days. The process shows no morphological or histochemical differences compared with that described in the previous sections. There is, however, a broadening of the zone of transition between the squamous and keratin layers. In this site there is an accumulation of sulphydryl, which is subsequently reduced in the keratin layer. The amount of reduction is less than is seen elsewhere. This is attributed to the rate at which this cyclical process takes place. It has also been studied in animals which bear transplantable hormone secreting tumours which were originally isolated from mouse pituitary glands. One group secretes thyrotrophin and, as a side effect, produces stimulation of the gonads. There is, however, no alteration in the oestrus cycle in this group. Two strains of adrenotrophic tumour were also studied. The presence of these tumours inhibits the periodic keratinisation of the vagina. This can be completely restored by adrenalectomy. It is considered that the process is probably the result of an inhibition of gonadotrophic hormones of the pituitary with the subsequent fall in the output of ovarian oestrogens.

P A R T V

THE FORMATION OF KERATIN IN TISSUE CULTURE

INTRODUCTION

The fundamental purpose of tissue culture is the maintenance of tissues alive outside the body. The methods fall into two main categories:- (a) where the aim is the rapid growth of cells usually of the same type. This is usually referred to as "tissue" or more correctly "cell" culture, (b) where tissue is maintained under conditions of slower growth in the hope of obtaining organisation of incompletely differentiated tissues, or the maintenance of organised structure in tissues already differentiated. This is usually referred to as "organ" culture. In general, two separate and distinct processes are involved, namely, proliferation which implies the reduplication of similar types of cells and differentiation which leads to the production of cells which are generally of a different type and may possess a more highly developed functional capacity. These two processes in vitro, as in the intact animal, are, if not mutually exclusive, generally exhibited in reverse relationship to each other.

In the present study both types of culture have been explored. It has been pointed out by Fawcett (1955) that various cell types in adult tissues vary greatly in their ability to grow and regenerate, and, therefore, some knowledge of the proliferative capacity of a particular cell type in vivo is a guide to what may be reasonably expected of that cell in vitro. The epidermis and

possibly squamous epithelium in general can be classified amongst those tissues which have a brief life span. They are replaced by the proliferation of relatively undifferentiated germinal elements. Accordingly it may be expected that, while the germinal cells proliferate in culture, the more highly differentiated cells survive for only a brief period.

It may also be expected that the most informative results are found in the type of culture which shows slow but organised growth. Here the process of keratinisation may proceed in a manner comparable to that observed in vivo. As will be discussed later, this has been shown to be the case. On the whole, cell growth has been unsuccessful. A brief description is included since the behaviour of cells growing out from squamous epithelium is interesting in relation to the findings with the organised type of growth as well as to other work in this field.

CELL CULTURE OF SKIN AND OTHER EPITHELIAL TISSUES

As indicated above, interest in this type of culture is primarily concerned with the cellular outgrowth from a piece of tissue and the morphological details of the outgrowing cells.

Since the method is described in detail in the technical appendix (p.294), only an outline will be given here. Fragments of tissue approximately 2 mm. in diameter are fixed to a coverslip by a plasma clot composed of chick embryo extract and chick plasma. The coverslip is placed in a test-tube containing medium and incubated at 37°C in the horizontal position. The media utilised include SM 199, and lactalbumin hydrolysate with various concentrations of human serum and chick embryo extract. The details are given in table XIV (p.188). The tissues cultured include human, mouse and chick skin, and a number of human skin tumours. The details and numbers of each type are given in table XV (p.189).

Results: As is shown in table XV growth by this method is poor. The explant of human adult skin remains unchanged for the first 12 hours. Subsequently in the cultures classified as showing good growth, small groups of cells extend out from the explant and generally extend over the whole of the coverslip in 3-4 days. The cells are mainly fibroblasts (figure 57, p.242) but in a few cultures small groups of epithelial-like cells are found (figures 58-59, pp. 243, 244).

Unlike the fibroblasts they form broad sheets of cells. They have large nuclei with distinct nuclear membranes and frequently prominent nucleoli. Mitotic figures are a prominent feature. The cytoplasm is eosinophilic and homogeneous. Intercellular bridges are not seen and sex chromatin is not identifiable. In short, the cells do have a resemblance to those of squamous epithelium. When they degenerate they do so by a process similar to that followed by the fibroblasts. They become detached from the glass, are rounded and their cytoplasm appears more eosinophilic and often contains small fat-like droplets. The cell membrane ruptures and only nuclear debris remains. At no stage of the process is there any morphological evidence of keratin formation (figure 60, p. 245).

Growth was considered to be good in only 2 cultures of human adult skin. Both were subcultured on 10 fresh coverslips after six days growth and subsequently at weekly intervals (figure 61, p.246). The cells began to lose their previous rather distinctive method of growth after the first transplant. They also became elongated and the nuclei became larger and more vesicular. Within 3 subcultures they had assumed "terminal cohesion" and acquired the morphological features of fibroblasts. By the fifth transplant they grew readily on glass and on morphological grounds were completely indistinguishable from established strains of fibroblasts which were being carried in the laboratory at the same time (figure 62, p.247).

The studies with foetal skin of animal origin were most satisfactory from the aspect of cell outgrowth. The outgrowths consisted of a mixture of fibroblasts and cells which showed epithelioid features. When these outgrowths were subcultured, however the end result was the same. Growth became luxurient and the cultures were rapidly overgrown by fibroblasts. Even those cultures which appeared, initially, to be pure growths of epithelioid cells, completely altered their morphological features and mode of growth and were again indistinguishable from fibroblasts within 2-3 generations. Growth of the human skin lesions was spasmodic and even in the few outgrowths which it was possible to subculture there was rapid alteration to the morphology typical of fibroblasts. None of the cultures showed at any stage any morphological evidence of keratin formation. Therefore, there seemed to be good practical and theoretical reasons for abandoning this interesting but, for the present purpose, relatively unrewarding approach. Attempts were made to establish organ cultures of skin. These will be described in the following section.

ORGAN CULTURE OF SKIN AND OTHER EPITHELIAL TISSUES

A. THE DEVELOPMENT OF THE METHOD

Interest in this type of culture is directed towards the behaviour of the explant and not the outgrowth of cells derived from it. Conditions accordingly, are adjusted to provide sustained survival of the tissue with a minimal outgrowth of cells. In the present investigation organised growth with differentiation of tissue to form keratin was sought.

The method adopted is described in full in the technical appendix (p.294) but as it is of considerable importance to the subject under study, a brief survey of its development will be given.

Organ culture as it is employed today, originated in the Strangeways Laboratory, Cambridge, originally under the direction of Strangeways (Strangeways & Fell, 1926), and pursued subsequently by Fell and her associates, who have studied the behaviour of limb rudiments and various embryonic tissues, including chick skin in culture (Fell 1951, 1953). Their technique is to place small pieces of tissue in a plasma clot in a watch glass which is surrounded by a moist chamber. While they have obtained excellent results with this method, it has certain disadvantages. It was found in preliminary experiments, that the tissue is surrounded by an outgrowth of fibroblasts which tends to liquefy the clot and as

a result the tissue sinks to the bottom of the dish. Likewise the semi-solid nature of the medium renders it difficult to add reagents to it and to be certain that they reach the cultured tissue. A further disadvantage is that flat tissues, such as skin, tend to become everted and form cysts, thus interfering with normal development. The numbers and types of preliminary cultures set up by this method are given in table XVI, (p.190).

In an attempt to utilise a fluid medium which offers many advantages, various devices have been used to support the tissues. They included tantalum foil and stainless steel (Trowell, 1959) and lens paper which floated in the medium (Chen, 1954). The above procedures were tried and none proved to be completely satisfactory. The use of sponge in cell culture has been described by Leighton (1951). His method was designed for the outgrowth of cells and not suitable for the present study. It did show however, that cellulose acetate sponge did not interfere with the growth of cells.

After considerable preliminary work the following method was found to permit the organised growth of tissue with the use of simple inexpensive equipment. While requiring some technical skill and dexterity, the method is essentially simple and reproducible results are readily obtained.

B. METHOD

Technical details are given in the appendix (p.295). Pieces of tissue 2-4 mm. square are dissected under the x 10 magnification of a dissecting microscope and placed on a strip of lens paper which rests on a block of cellulose acetate sponge in a flat-bottomed watch glass. Medium is added to the watch glass and fed to the lower parts of the tissue by the lens paper which acts as a wick, (figure 63, p. 248). To reduce loss of fluid by evaporation and assist in the maintenance of the gaseous phase, the culture dishes are placed in a specially designed 'Perspex' container (figure 64, p. 249), which is flushed twice daily with 5% CO_2 in oxygen. The cultures are incubated at 37°C and the medium changed at intervals as described in the text.

C. CULTURE MEDIA

Considerable preliminary work was also necessary to establish the most suitable type of medium. A list of the various media tried initially is given in table XVII (p. 191). The various tissues cultured are also given in table XVIII (p. 192). It was found that synthetic chemical media were adequate for the production of keratin from squamous epithelium. The addition of serum and embryo extracts produced active growth of undifferentiated cells which did not form keratin. Such a medium SM 199 (Morgan, et al. 1950) was available commercially and was used routinely thereafter.

Its composition is given in table XIX (p. 193).

D. EPITHELIAL TISSUES STUDIED

As will be described later, this method supports the in vitro formation of keratin by various types of epithelium. These include foetal and adult human skin, foetal mouse skin, infantile and adult mouse vagina. The total numbers of cultures of each type of tissue are given in table XX (p.194). Substances which might be expected to affect the process of keratinisation have also been incorporated in the media. These include vitamin A, squalene, oestradiol and other steroids. Details of the amounts of the substances added and of cultures in each group are given in tables XXI-XXV (pp. 195-199). The morphology and histochemistry of the process in each type of epithelium is described in a separate section. To avoid unnecessary repetition, the process in human foetal and adult skin will be described in some detail but in the subsequent sections dealt with more briefly except where it differs from the findings in the human material.

ORGAN CULTURE OF HUMAN FOETAL SKIN

A. MATERIAL

Through the co-operation of Dr. D. MacIntosh a portion of skin 1 x 1.5 cm. was received from the back of a viable 13 week human foetus. Cultures were set up by the standard method within two hours of the hysterotomy. The numbers of cultures in each group are detailed in table XXI (p. 195).

B. RESULTS

Normal Histology: As shown in figure 65 (p. 250) at 13 weeks human skin consists of an oedematous dermis in which there are thin walled blood vessels and fibroblasts. It is covered by an epidermis which consists of a basal S. germinativum composed of a single layer of cuboidal cells and a S. intermedium of 2-3 layers of large vacuolated cells with relatively small, generally eccentric nuclei. These cells contain abundant glycogen. Superficially there is a thin layer of periderm consisting of flattened eosinophilic cells. There is no reaction in any layer for either sulphydryl or disulphide.

Cultures in medium 199 alone: After 24 hours in culture few changes are seen. The dermis is more oedematous and cellular. In the epidermis the changes are also slight. The S. germinativum is no longer regular and its cells are taller. The S. intermedium now

consists of 3-4 layers of cells in which the cytoplasm is more oedematous and the nuclei smaller and now central.

By 48 hours, while the dermis is relatively unchanged, the epidermis bears a distinct resemblance to that of adult tissues. It comprises a prominent basal layer of tall columnar cells with large central nuclei. A moderate number of mitotic figures is present. The S. intermedium now consists of 4-5 layers of cells. Immediately above the basal cell layer the cells are cuboidal, but as they approach the surface they become more flattened and the outermost layer is distinctly ovoid. The cytoplasm is now faintly eosinophilic but still unreactive for sulphydryl. The nuclei are still relatively large, central and basophilic. Mitotic figures are very infrequent and nucleoli are not seen. Superficially the periderm, which is swollen and becoming detached from the underlying S. intermedium, contains a few pyknotic nuclei. Keratohyalin granules are not seen and there is no morphological evidence of keratinisation.

At 4 days there is a distinct resemblance to adult epidermis. The basal cell layer now consists of a single layer of densely packed tall columnar cells. They contain a moderate amount of RNA but are unreactive for glycogen, sulphydryl or disulphide. The cells of the S. intermedium which are now similar in appearance to adult squamous cells, contain a moderate amount of glycogen and are

faintly reactive for sulphydryl. Superficially there is now a fairly dense band of eosinophilic material which is refractile and has the tinctorial characteristics of keratin. It is moderately reactive for sulphydryl and gives a faint reaction for disulphide. In places the periderm has been completely lost but elsewhere it is still loosely attached to this keratin-like material.

By 8 days in this medium the skin is almost indistinguishable from normal mature adult epithelium (figure 66, p. 250). The dermis shows no notable changes from those in the second day cultures. There are no interpapillary processes but the dermo-epidermal boundary is sharply defined by a single layer of cuboidal cells. Above, there are 3-4 layers of typical squamous cells. Superficially there is a thin well formed keratin layer in which there are isolated areas of parakeratosis (figure 67, p. 251). Keratohyalin granules are not a prominent feature and there is no distinct S. granulosum. Histochemically the keratin layer is similar to that in adult skin except for a slightly higher level of sulphydryl and less demonstrable disulphide. There is also some broadening of the sulphydryl rich transition zone between the squamous and keratin layers.

Cultures in medium 199 with oestradiol: At no stage is there any significant morphological or histochemical difference between these cultures and those in the control medium. The process, however,

is more rapid, i.e., the end-point of keratin formation is achieved earlier.

Thus, although indistinguishable after 2 days from the control preparations, these cultures by 4 days resemble the stage reached by the controls after 8 days in culture. A distinct layer of keratin is present and is covered, in places, by the remains of the periderm. Although there are a few pyknotic nuclei there is no evidence of keratohyalin granules. By the eighth day the cultures show early degenerative changes, particularly in the dermis. In the epidermis which is now poorly demarcated from the dermis, the basal cells are vacuolated and the squamous cells are smaller. Their nuclei show some irregularity in size and shape. The cytoplasm of many cells is now vacuolated and some contain eosinophilic droplets which have the morphological features of keratin. Dyskeratotic cells are numerous throughout the squamous layer and superficially there is a loosely attached layer of densely staining keratin.

Cultures in Medium 199 with Vitamin A: Compared with those in medium alone, the changes in medium with added vitamin are striking. Apart from slight oedema of the dermis there are no significant changes after 24 hours. Thereafter consistent changes develop. The dermis gradually becomes more eosinophilic and highly cellular. Many of the cells appear to be young fibro-

blasts but others are smaller and resemble immature lymphocytes. By 4 days the epidermis has become thicker, comprising 4-5 cell layers. The basal cell layer is now irregular and consists of 2-3 layers of cuboidal cells with scanty eosinophilic cytoplasm and relatively small central darkly staining nuclei. A few cells are in mitosis. These cells are moderately reactive for RNA, but contain scanty glycogen and no sulphydryl or disulphide. The more superficial of these apparently actively proliferating "basal" type cells have slightly more cytoplasm which is more deeply eosinophilic and contain more glycogen. They are covered by 2-3 layers of cells of squamous appearances, which contain less RNA, more glycogen and are faintly reactive for sulphydryl. At 8 days both these layers are increased in thickness. The more superficial contain some keratohyalin granules and they are covered by a single layer of flattened cells (figure 68, p. 252). These cells are eosinophilic and give a slightly more intense reaction for sulphydryl. They are however, not refractile and unreactive for disulphide.

C. SUMMARY

Human skin from a 13 week foetus develops in synthetic medium 199 from its primitive structure to multilayered stratified squamous epithelium with the formation of keratin. This is complete within 8 days. There are no morphological or histochemical features which distinguish the process from that seen in normal human skin, apart from a broadening of the transition zone between the squamous and keratin layers. This is demonstrable by the increased staining for sulphydryl in the upper squamous layer and the relatively slight decrease in concentration in the keratin layer. This corresponds with the appearance of demonstrable disulphide. Cultures set up in medium containing 50 µg. of oestradiol reach the same degree of development in 4 days instead of 8. Otherwise the process is essentially similar as regards morphology and histochemistry. Culture in medium containing 500 µg. vitamin A show proliferation of the basal cells but no definite evidence of keratinisation although by 8 days there is a thin superficial layer of partially keratinised cells. In these cultures there is some accumulation of glycogen but little sulphydryl and a complete absence of disulphide.

ORGAN CULTURE OF HUMAN ADULT SKIN

A. MATERIAL

Excess skin, removed in the Plastic Surgery Unit for grafting purposes, was used. It was generally taken from the lower abdomen or anterior aspect of the thigh. There was no significant difference in the histology of the various specimens received. The appearance of such a specimen is shown in figure 69 (p. 253). It consists of whole thickness, i.e., epidermis and a portion of the upper dermis. The histochemical findings are as described in Part I (p. 15). The number of cultures in each group and the amounts of the various substances added to the medium are given in table XXII (p.196).

B. RESULTS

Cultures in medium 199 alone: Apart from complete desquamation of the superficial layer of keratin, the changes after 24 hours in culture are relatively slight. The basal cell layer is still regular but a few cells are vacuolated. The squamous cells are now less closely packed and again a few of these are vacuolated. In the more superficial layers an occasional cell shows increased cytoplasmic eosinophilia. This is associated with a slightly increased reaction for sulphydryl. The S. granulosum is no longer present as a distinct layer and its place is now occupied by a fairly dense band of eosinophilic keratin-like material.

After 2 days more striking changes are present. The basal layer is irregular and the lower part of the epidermis consists of 3-4 layers of large ovoid or spindle-shaped cells which have basophilic granular cytoplasm. The nuclei are still "squamous" in type and often contain eosinophilic nucleoli. The cells appear loosely attached to each other and in places there is separation of the epidermis from the underlying tissues (figure 70, p.254). These cells which contain scanty RNA and glycogen are slightly reactive for sulphydryl. More superficially there is a fairly sharp transition to a zone where the cells have become rounded and where their nuclei shrink rapidly and become densely basophilic. At the upper surface the cells often have no nuclei and are loosely attached to each other and to the superficial strands of deeply eosinophilic keratin which cover the lesion. As the surface is approached this zone shows a gradual increase in the amount of sulphydryl and the appearance of a reaction for disulphide.

At 4 days the process is an extension of that described above. The lower epidermis comprises a 2-4 cell layer of elongated cells with large vesicular nuclei and in which there are a moderate number of mitotic figures. Above these cells there is a loosely attached zone which is composed largely of parakeratotic cells and which gives a moderate reaction for sulphydryl and a faint reaction for disulphide. Superficially there are laminated strands of deeply eosinophilic keratin. Keratohyalin granules are not present

(figure 71, p. 255).

At 6 days the appearances are only slightly different from those described above. The cells at the base of the epidermis now more closely resemble fibroblasts. Above them there is a fairly thick layer of apparently more fully developed keratin in which there are fewer parakeratotic cells. In this zone the reaction for disulphide is now more intense and that for sulphydryl decreased. The absence of keratohyalin granules is a notable feature (figure 72, p. 255).

Cultures in medium 199 with oestradiol: In general, as with the foetal skin, there are no significant differences in the various morphological stages which the cultures undergo, and the common end-point is that of complete conversion of the existing squamous layer to keratin. The process, however, again appears to be accelerated, although this is not so pronounced. Thus at 24 hours the cultures show little change. In the highest concentration of oestrogen (50 µg/ml. of medium) the squamous cells are rather more swollen than in the other concentrations or the control preparations at the same period. Otherwise there are no detectable differences between the various concentrations employed.

The changes found at 4 days in the control media are well established in all concentrations by 2 days (figure 73, p. 256). Thereafter the changes are similar and the end point of complete devitalisation of the squamous cell layer is complete by 6 days

(figure 74, p.256). There is of course, active proliferation at the dermo-epidermal junction of large spindle shaped "epithelioid cells". Only two slight differences are noted with any degree of regularity. (1) The keratin formed appears to be much more dense and consists of a solid mass of uniformly eosinophilic material. (2) Parakeratotic cells are more numerous and occur in groups, especially in the mouths of hair follicles and at the edges of the culture (figure 75, p.257).

Cultures in medium 199 with vitamin A: The findings in the cultures with added vitamin are also less striking than those found in the cultures of foetal skin. After 24 hours all the cultures are essentially similar and show no remarkable difference when compared with normal skin, with the exception of the highest concentration, (1000 µg/ml. of medium). Here, cleft-like spaces form and separate the dermis and epidermis. Within 2 days the epidermis is generally completely shed and the remaining epithelium consists of a double layer of small darkly staining cells of basal cell type. These cells remain virtually unchanged even after 8 days in culture. They show no evidence of mitotic activity.

The changes in the other concentrations are essentially similar. Thus, by 2 days there is slight patchy separation at the dermo-epidermal junction. Elsewhere there is some slight "basal" cell proliferation. This is more pronounced by 4 days but the lower squamous layer is unchanged (figure 76, p. 258). The upper two

squamous layers, however, now form a syncytial-like mass in which there are small pyknotic nuclei. This zone forms a band of moderately intense staining for sulphydryl. This keratin-like material merges into the more eosinophilic and dense superficial pre-formed keratin layer, which in these cultures does not desquamate. By 6 days the features are largely similar to those in the control medium but some cells are now vacuolated (figure 77, p. 258) and the amount of keratin formed not so abundant. Keratohyalin granules are not a prominent feature although in places isolated collections occur (figure 78, p. 259). By 8 days the cultures are indistinguishable from those in medium 199 alone.

Cultures in medium 199 with oestradiol and vitamin A: no morphological or histochemical differences can be detected between these cultures and those which contain oestradiol alone.

Cultures in medium 199 with dehydroisoandrosterone: At no stage are there any morphological or histochemical differences between these cultures and those set up in medium containing oestradiol. Therefore, compared with the controls they show no essential differences in the stages through which they proceed to final keratinisation. There is however, acceleration of the whole process.

Cultures in medium 199 with squalene: Complete separation of the epidermis from the dermis occurs in the two highest concentrations (1000 and 100 µg/ml. of medium). In the other two concentrations

there is some increase in thickness of the squamous cell layer and some "basal cell" proliferation. Compared with the controls there is slight retardation of the process of keratinisation. By 6 days however, the process is indistinguishable from that in the controls.

C. SUMMARY

Human adult skin cultured in synthetic medium 199 alone continues to form keratin. The greater part of the squamous cell layer is converted into keratin within 4 days. The basal cells are replaced by actively proliferating cells of squamous type. Histochemically the process is associated with a decrease in the amount of glycogen and sulphydryl. The latter diminishes as keratinisation proceeds with a corresponding appearance of disulphide. A similar process takes place in cultures which contain 50-0.05 µg./ml. of oestradiol or dehydroisoandrosterone. It is however, accelerated and the same stages in the process are reached in approximately half the time taken by the control cultures. Cultures to which are added 100-1 µg./ml. of vitamin A show very slight retardation in the formation of keratin. There is also some proliferation of basal cells. Cultures which contain 10 and 1 µg./ml. of squalene also show some retardation of the process. Cultures which contain both oestradiol in concentrations ranging from 50-0.05 µg./ml. and 50 µg./ml. of vitamin A acetate, show no difference when compared with those containing similar

concentrations of oestradiol alone.

ORGAN CULTURE OF FOETAL MOUSE SKIN

A. MATERIAL

Embryos were obtained by breeding stock white mice of indeterminate origin. The animals were killed by ether and the uterus removed with aseptic precautions. The embryos were washed in medium 199 and pieces of skin measuring 6 x 4mm. removed from their backs. After removal of the subcutaneous fat, 2 mm. squares were cultured by the standard method. The numbers of cultures in each group and the amount of the various substances added to the medium are given in table XXIII (p.197).

B. RESULTS

Normal Histology: The development and structure of foetal mouse skin resembles human tissue in many aspects. Thus, at 12 days the epidermis consists of a basal layer of relatively large tall cuboidal cells with large central deeply basophilic nuclei (S. germinativum). Above this there are 2-3 layers of large oval cells in which there are similar nuclei and abundant pale eosinophilic cytoplasm. These cells contain abundant glycogen, (S. intermedium). Superficially there is the periderm in which there are pyknotic nuclei, (figure 79, p.260). By about the 15th day it has become prominent and more eosinophilic. The S. intermedium has increased to 4-5 cell layers in thickness and is now distinctly squamous in type. These cells contain less glycogen and are now faintly

reactive for disulphide. Superficially a few keratohyalin granules are present. By the 18th day the periderm has almost completely disappeared and forms a thin layer on top of a broad zone of well formed keratin. The S. intermedium is now composed of typical squamous cells, and a broad S. granulosum is now present (figure 80, p.260). The basal cell layer is irregular and poorly demarcated from the underlying dermis.

From the above brief description it emerges that keratinisation of foetal mouse skin is well established by the 18th day. Therefore the cultures of this tissue will be considered in two groups; (i) cultures from embryos of this age or older, i.e., a group where keratin is already formed and (ii) cultures from younger embryos, generally 12 day, where keratin is not already present.

(i) Culture of keratinised skin

Cultures of keratinised skin in medium 199 alone: No notable changes appear until 2 days. By then the keratin layer is looser and largely detached. The S. granulosum is less regular and thinner in places. In the squamous layer the cells are larger and the basal cells also larger and more squamous in appearance (figure 81, p. 261). At the edge of the culture these cells become elongated and grow along the undersurface (figure 82, p.262). They are moderately rich in glycogen but almost completely unreactive for sulphydryl. After 4 days in culture the pre-formed keratin is detached and the surface is formed by a thin

layer of more densely eosinophilic keratin which is moderately reactive for sulphydryl and gives a faint reaction for disulphide. The squamous cells which are larger and show some pleomorphism now almost completely surround the specimen (figure 83, p. 263). By 6 days there is a largely necrotic dermis surrounded by 4-5 layers of squamous cells which are again more pleomorphic and show moderate mitotic activity. They contain abundant glycogen and are almost completely unreactive for sulphydryl. More superficially they merge into an eosinophilic syncytium in which there are numerous nuclear fragments (figure 84, p. 264). This zone gives a fairly intense reaction for sulphydryl and a faint reaction for disulphide. By 8 days the whole specimen is largely infiltrated by actively growing cells which although pleomorphic are still squamous in type. Superficially there are loosely attached fragments of keratin (figure 85, p. 264).

Cultures of keratinised skin in medium 199 with oestradiol: The highest concentration (50 µg./ml.) consistently produces degenerative changes within 24 hours and subsequently complete separation of the epidermis. In the other concentrations there are no essential differences between these cultures and the controls although there is an acceleration of the process. Thus by 2 days the pre-formed keratin layer is largely detached (figure 86, p.265) and the squamous cells are larger and extend into the underlying dermis (figure 87, p.266). By 4 days there is a wide layer of densely eosinophilic

keratin in which there is considerable sulphydryl but only an extremely faint reaction for disulphide. Thereafter the cultures are indistinguishable from those in medium 199 alone.

Cultures of keratinised skin in medium 199 with dehydroisoandrosterone The two highest concentrations (50 and 5 µg./ml.) consistently produce degenerative changes within 24 hours. In the other concentrations the findings are essentially similar to those with oestradiol, i.e. there is an acceleration of keratinisation but no alteration in the various stages of the process.

Cultures of keratinised skin in medium 199 with vitamin A: The highest concentration of added vitamin (1000 µg./ml.) consistently produces extensive separation of the epidermis which is complete within 2 days. The findings in the other 3 concentrations are similar and differ little from the controls. At 4 days the preformed keratin is still attached and beneath it there is a narrow zone of much more dense material. This contains a higher concentration of sulphydryl and is less reactive for disulphide. Keratohyalin granules are a prominent feature. By 6 days the only detectable difference in these cultures is in the keratin layer where the preformed keratin is still attached to the underlying layer which has been formed in culture.

Cultures of keratinised skin in medium 199 with oestradiol and vitamin A: There is relatively little alteration compared with the cultures in oestradiol alone, except that there is often rapid and

extensive separation of the epidermis in the two highest concentrations of the steroid (50 and 5 µg./ml.).

(ii) Culture of non-keratinised skin

Cultures of non-keratinised skin in medium 199 alone: After 1 day there is considerable increase in the cellularity of the dermis. The cells are almost all fibroblasts. The epidermis shows some separation of the periderm and increase in the thickness of the S. intermedium. By 2 days the epidermis is distinctly squamous, and the basal cell layer is no longer composed of distinctive cells. Demarcation between the epidermis and dermis is now indistinct. There is a prominent S. granulosum and a thin layer of pale eosinophilic keratin which reacts intensely for sulphydryl but is unreactive for disulphide. At 4 days the epidermis consists of apparently mature squamous epithelium which is covered by a thick layer of laminated keratin (figure 88, p.267). The squamous epithelium is moderately reactive for sulphydryl and this reaction is more intense in the keratin layer where there is also a faint reaction for disulphide. By 6 days the dermis is largely necrotic and completely surrounded by hyperplastic squamous epithelium in which the cells are pleomorphic and show considerable mitotic activity. Superficially these cells are vacuolated and merge into a broad zone of loose eosinophilic strands of keratin. This zone is moderately reactive for sulphydryl and this becomes less

intense as the outermost layers are reached. This diminution in the intensity of staining for sulphydryl is accompanied by a corresponding increase in that for disulphide.

Culture of non-keratinised skin in medium 199 with oestradiol:

No appreciable differences are noted between any of the concentrations apart from the highest (50 µg./ml), which frequently shows complete separation of the epidermis. The cultures are similar to those in medium 199 alone but slight differences are present. Thus at 2 days the dermis is more cellular. The thickness of the squamous layer is unchanged but the basal cells still retain their distinctive morphology. A few of the squamous cells are vacuolated and the keratin layer is thicker, more deeply eosinophilic and contains parakeratotic nuclei. In this layer the concentration of sulphydryl is lower and that of disulphide higher than in the control cultures in medium alone. At 4 days the appearances are similar to the controls. There is, however, increased cellularity of the squamous layer in which many of the cells are pleomorphic. At 6 days they form fairly dense collections of actively growing cells which surround the cultures and extend into the dermis (figure 89, p.267). The most superficial layer of well formed keratin has been shed and the cultures are now covered by a broad zone of pale eosinophilic keratin in which there are numerous parakeratotic cells. This zone is moderately reactive for sulphydryl and only faintly so for

disulphide.

Cultures of non-keratinised skin in medium 199 with dehydroisoandrosterone: The highest concentration (50 µg./ml.) produces degenerative changes and no further development of the epidermis occurs. The findings in the other concentrations are essentially similar to those found with oestradiol.

Cultures of non-keratinised skin in medium 199 with vitamin A: At 2 days there are striking differences when the cultures are compared with the controls. The epidermis is now 10-12 layers in thickness. In the lower layers the cells are much smaller and are generally cuboidal. In the more superficial layers they are larger and contain a moderate amount of glycogen and are faintly reactive for sulphydryl. Although there are a moderate number of keratohyalin granules, in the more superficial cells, there is no evidence of keratinisation at this stage. At 4 days the cells are less closely packed and are more distinctly squamous in type. They contain less glycogen and more sulphydryl. An extremely thin layer of eosinophilic keratin-like material is present in places (figure 90, p.268). At 8 days there is a well formed layer of keratin and the squamous layer is reduced to a thin zone of actively proliferating spindle-shaped cells. There is widespread separation and most of the basal cells are pyknotic (figure 91, p.268).

Cultures of non-keratinised skin in medium 199 with squalene: The 3 highest concentrations of squalene frequently produce degenerative changes. The findings in the lowest concentration (1 µg./ml.) are essentially similar to those in the cultures with added vitamin A.

Cultures of non-keratinised skin in medium 199 with vitamin A and oestradiol: Only degenerative changes are produced in the two highest concentrations of steroid. In the lowest (500 µg. of vitamin A plus 0.5 or 0.05 µg./ml. of oestradiol) there is some slowing of the process of keratinisation. Thus at 4 days the findings are similar to those in medium 199 alone. By 6 days however, they are again similar to those in oestradiol alone.

C. SUMMARY

Two groups of tissue have been studied, (i) skin from embryos of 18 days gestation in which the keratin layer is well formed and (ii) from younger embryos in which there is no pre-formed keratin. In the keratinised group the squamous layer continues to form keratin with the detachment of that already present. New keratin is formed within 4 days. The process is associated with an increased concentration of sulphydryl in the transition zone which diminishes in the more superficial layers as the concentration of disulphide increases. The addition of 5-0.05 µg./ml. of oestradiol or dehydroisoandrosterone accelerates but does not alter the process. 100-1 µg./ml. vitamin A in medium alone or in medium with 0.5-0.05 µg./ml. oestradiol has little effect on the cultures in this group. The changes in the cultures without pre-formed keratin are more striking. Within 2 days the epithelium is squamous in type and by 4 days there is a well formed layer of keratin which is considerably increased by 6 days. The process is accelerated by the addition of 5-0.05 µg./ml. oestradiol or dehydroisoandrosterone and retarded by the addition of 1000-1 µg./ml. vitamin A or 1 µg./ml. squalene. The histochemical findings again show the process to be associated with a decrease in demonstrable sulphydryl and an increase in disulphide.

ORGAN CULTURE OF INFANTILE MOUSE VAGINA

A. MATERIAL

Vaginas were taken from stock white mice aged 3-7 days. The bladder and urethra were removed *in situ* with the aid of a dissecting microscope. After washing in medium 199 the uterine horns, cervix and outer squamous portion were removed. The remaining vaginal tissue was slit longitudinally and cut into 5 strips. One piece was retained for histological examination and the others set up in culture by the standard method. The number of cultures in each group and the amounts of the various substances added to the medium are given in table XXIV (p.198).

B. RESULTS

Normal histology: Vaginas at this stage comprise from without inwards, (i) a flattened layer of large endothelial-like cells, (ii) a broad highly cellular zone in which there are thin-walled blood vessels and (iii) the epithelial layer. This consists of 2-3 layers of closely packed large cuboidal cells. The cytoplasm which is relatively scanty, is eosinophilic and granular. The nuclei which are large, central and basophilic often contain nucleoli. Superficially, there is a single layer of flattened cells (figure 92, p. 269).

Cultures in medium 199 alone: After 24 hours the epithelium is more

sharply demarcated from the underlying tissues. It now consists of 3 layers of low cuboidal cells. The superficial flattened layer has disappeared. After 2 days, while much of the epithelium is unchanged, there are areas which are up to 10 cell layers in thickness. The more superficial cells are still cuboidal but beneath them there are large cells of squamous type. These contain abundant RNA and glycogen but are unreactive for sulphydryl or disulphide. The basal layer is now indistinct (figure 93, p.270). After 4 days in culture the epithelium is 4-5 cells thick and distinctly squamous in type (figure 94, p.271). In some areas there is a thin layer of well formed keratin and scanty keratohyalin granules are present (figure 95, p.272). By the 8th day the culture is covered by a thick layer of dense keratin in which there are parakeratotic cells. In places there are degenerate cuboidal cells still attached (figure 96, p.272). There is a moderate reaction for sulphydryl in the squamous layer which is intensified in the transition zone. A slightly less intense reaction is present in the keratin layer where there is now a faint reaction for disulphide.

Cultures in medium 199 with oestradiol: These cultures again show no significant or histochemical features when they are compared with the controls. There is however, acceleration of the process of keratinisation. Thus by the 4th day the epithelium, which is now typically squamous, is covered by a distinct band of dense

acellular keratin (figure 97, p.273). At 6 and 8 days the cultures show merely a thicker and more dense layer of keratin which shows a higher concentration of disulphide than the cultures in medium alone.

Cultures in medium 199 with testosterone: At 2 days there is still no squamous differentiation of the cuboidal cells. They are, however, larger and contain abundant RNA and glycogen. There is no reaction for sulphydryl or disulphide. At 4 days the epithelium is thicker and generally consists of 6 layers. Superficially there is a flattened layer of cells. Beneath them the cells are cuboidal or oval. They have faintly eosinophilic granular cytoplasm and large central nuclei. The appearances suggest partial squamous development (figure 98, p. 273). They contain scanty glycogen and less RNA than the cuboidal cells but are also unreactive for sulphydryl or disulphide. By 6 days the whole epithelial layer is composed of similar cells. In many cells the cytoplasm is more eosinophilic and faintly reactive for sulphydryl. In places there are scanty parakeratotic cells (figure 99, p. 274). By 8 days the epithelium is distinctly squamous in type and intercellular bridges can be seen between some cells. The cells now have scanty glycogen and RNA but are now moderately reactive for sulphydryl. Superficially there is a thin layer of keratin which contains less sulphydryl and is faintly reactive for disulphide.

Cultures in medium 199 with oestradiol and testosterone: In the highest concentration of steroid (50 µg./ml. of both oestradiol and testosterone) the cultures rapidly become necrotic. The other changes are essentially similar to those found with oestradiol alone but the process is retarded. Thus at 4 days the changes are similar to those seen at 2 days in medium with oestradiol alone. By 6 days, however, these two groups are indistinguishable.

Cultures in medium 199 with corticosterone: The cultures remain unchanged for 2 days. By 4 days the epithelium is 4-5 cell layers in thickness and still composed of cuboidal cells. The superficial cells are swollen and less regular, and the lower cells show slight squamous differentiation. By 6 days the epithelium is 3-4 cells thick and distinctly squamous in type. There is reduction in the amount of glycogen. The reaction for sulphydryl is moderately intense with denser staining in the transition zone. By 8 days there is a thin layer of keratin in which the reaction for sulphydryl is still intense and only slightly less than that in the underlying transition zone. It gives a faint reaction for disulphide.

Cultures in medium 199 with vitamin A: Cultures with the highest amount of added vitamin (1000 µg./ml.) show degenerative changes with separation of the epithelium. In the other concentrations the epithelium is unchanged at 2 days. After 4 days it has increased in thickness to 4-5 cell layers. The superficial cells are cuboidal

and rich in glycogen but unreactive for sulphydryl or disulphide. The remainder of the epithelium consists of large cells which begin to resemble squamous cells. By 6 days the cells are distinctly squamous (figure 100, p. 275) and moderately reactive for sulphydryl. At 8 days there is a thin superficial layer of pale eosinophilic keratin. The reaction for sulphydryl is still moderately intense while there is only a faint reaction for disulphide.

C. SUMMARY

The cuboidal epithelium of the infantile mouse vagina becomes squamous during culture in medium 199. This transformation occurs within 4 days and by this time there is evidence of early keratin formation. This is well advanced by 6 days, and accompanied by a decrease in demonstrable sulphydryl and the appearance of disulphide. The addition of 50-0.05 µg./ml. oestradiol accelerates the process. This acceleration can be temporarily abolished by the addition of 5-0.5 µg./ml. of testosterone. 50-0.05 µg./ml. of testosterone, 10-0.01 µg./ml. corticosterone and 100-1 µg./ml. of vitamin A retard squamous differentiation and keratin formation. Although the rate of keratin formation can be altered there are no morphological or histochemical differences in the various stages of the process.

ORGAN CULTURE OF ADULT MOUSE VAGINA

A. MATERIAL

Vaginas were obtained from 12-15 week old white mice which were bred in this laboratory. After dissection the vaginas were washed in medium 199. The cervix and outer squamous portion were removed and the remainder cut into 5 strips. One strip was retained for histological examination and the others cultured by the standard method. The morphology and histochemistry of the adult vagina have already been described in part IV (p. 86). The oestrus cycle in these animals was determined by daily vaginal smears, and two groups were studied - (i) vaginas taken at oestrus when there already is a well formed keratin layer and (ii) at di-oestrus when there is no preformed keratin. The numbers of cultures in each group and the amounts of the various substances added to the medium are given in table XXV (p. 199).

B. RESULTS

Cultures in medium 199 alone: By the 2nd day the oestrus cultures show desquamation of the greater part of the keratin layer. The underlying epithelium is relatively unchanged apart from vacuolation of an occasional cell. Compared with the tissue before culture the squamous cells contain more RNA and less glycogen. A moderately intense reaction for sulphydryl is present and this is intensified

in the superficial partially keratinised cells (figure 101, p. 276). By 4 days the squamous layer is thinner and covered by a deeply eosinophilic layer of keratin. This layer is slightly reactive for sulphydryl and gives an intense reaction for disulphide (figure 102, p.276). At 6 days the changes are complete as no further alteration develops in similar cultures studied for 14 days. The epithelium now consists of 2-3 layers of flattened cells which are pleomorphic and contain frequent mitotic figures. They are rich in glycogen and RNA but only faintly reactive for sulphydryl. They are covered by a broad layer of loosely arranged strands of keratin in which there are nuclei in various stages of disintegration (figure 103, p. 277). More superficially the keratin becomes more dense and this is associated with a decrease in sulphydryl and an increase in disulphide.

In the cultures of vaginas at di-oestrus there is little morphological change at 2 days. There is less RNA and glycogen while the reaction for sulphydryl which is moderate throughout the squamous layer, is markedly intensified in the most superficial layers (figure 104, p. 278). By 4 days there is a distinct layer of superficial keratin which has less demonstrable disulphide and more sulphydryl than the oestrus cultures at the same stage. The squamous cells contain more RNA and glycogen is abundant in places (figure 105, p. 278). Keratohyalin granules are more prominent

and there are more parakeratotic cells. By 6 days the cultures are indistinguishable from the oestrus cultures.

Cultures in medium 199 with oestradiol: In the cultures at oestrus the highest concentration (50 µg./ml.) produces degenerative changes with separation of the epithelium. As in other sites the changes do not differ from those in medium 199 alone. There is, however, acceleration of keratin formation and this is more marked than in other tissues. Thus by 2 days the preformed keratin is largely detached and the greater part of the squamous layer is keratinised. This more rapidly formed keratin has a higher concentration of sulphydryl and less disulphide.

In the di-oestrus cultures the highest concentration (50 µg./ml.) is toxic and degenerative changes result. Again compared with the changes in medium alone there is an acceleration of keratinisation without any difference in the stages through which it progresses.

Cultures in medium 199 with testosterone: In the vaginas taken at oestrus the two highest concentrations (50 and 5 µg./ml.) produce degenerative changes. Thus at 24 hours the greater part of the squamous and keratin layers are desquamated (figure 106, p. 279). By 4 days the remaining epithelium is reduced to 2-3 layers of cuboidal cells (figure 107, p. 279) which by 6 days become flattened and grow out to encircle the culture. These cells are

rich in RNA and glycogen and slightly reactive for sulphydryl. At 8 days they are again more squamous in type and in places are covered by a thin layer of keratin. The cultures in the other concentrations (0.5 and 0.05 µg./ml.) show little alteration at 2 days apart from some desquamation of the preformed keratin layer. At 4 days the epithelium is still squamous in type. The two most superficial layers contain numerous parakeratotic cells although there is no evidence of keratin formation. By 6 days the epithelium is thinner and now covered by a thin layer of keratin which is only slightly less reactive for sulphydryl than the squamous layer. It is also faintly reactive for disulphide.

The cultures at <u>di-oestrus</u> are largely similar. The two highest concentrations again produce degenerative changes. Thus at 24 hours there is widespread desquamation of the greater part of the squamous epithelium. The subsequent behaviour of the cultures is similar to those at <u>oestrus</u>. In the remaining concentrations the cultures remain unchanged until 4 days when the epithelium is squamous in type and thicker than in the <u>oestrus</u> cultures at the same stage. It is also covered by a layer of parakeratotic cells but again there is no keratin formation (figure 108, p. 280). Thereafter the changes are similar and there is no keratin formation until 8 days.

<u>Cultures in medium 199 with oestradiol and testosterone</u>: In the highest concentration of steroid (50 µg./ml. of both oestradiol and

testosterone) in both oestrus and di-oestrus cultures there is complete separation of the epithelium and subsequent degenerative change. In the other concentrations, there is slowing of the process of keratinisation which is now similar in morphology and histochemistry to that in medium alone.

Cultures in medium 199 with progesterone: The findings with the oestrus vaginas are similar to those with testosterone. Thus, in the two highest concentrations (50 and 5 µg./ml.) there is separation of parts of the squamous layer (figure 109, p.281). The subsequent behaviour of the cultures in these groups, i.e., progesterone and testosterone, is indistinguishable. In the other concentrations (0.5 and 0.05 µg./ml.) there are slight differences. At 4 days the cells are less distinctly squamous in type. Many are vacuolated and contain more glycogen and less sulphydryl. By 6 days, while most of the cells are distinctly squamous, in some areas groups of vacuolated cells are still present. Keratin is not formed until the 8th day.

In the di-oestrus cultures, the changes in the two highest concentrations are identical to those with testosterone. In the other concentrations keratin formation is retarded. At 4 days the epithelium consists of 7-8 layers of closely packed cells. Many are vacuolated and contain pyknotic nuclei (figure 110, p.282). The superficial cells are swollen and parakeratotic cells are not

present. At 6 days the superficial cells are squamous in type (figure 111, p.283). There is no keratin formation at this stage. At 8 days, however, the appearances are indistinguishable from those with testosterone and a thin layer of keratin is present.

Culture in medium 199 with corticosterone: The morphological and histochemical findings in both oestrus and di-oestrus cultures are essentially similar to those in medium 199 with progesterone.

Cultures in medium 199 with vitamin A: In the oestrus cultures in the two highest concentrations of added vitamin (1000 and 100 µg./ml.) there is complete separation of the epithelium within 2 days. In the lowest concentrations (10 and 1 µg./ml.) at 2 days there is separation of the preformed keratin and parts of the squamous layer. By 4 days the remaining epithelium consists of 4-5 layers of cuboidal cells which are rich in glycogen but only faintly reactive for sulphydryl. The more superficial cells are flattened and contain keratohyalin granules (figure 112, p.284). By 6 days the cells are more distinctly squamous in type. Many are vacuolated and show various forms of nuclear degeneration (figure 113, p. 285). Keratin is not present. At 8 days the epithelium is composed of typical squamous cells. They are moderately reactive for sulphydryl and contain abundant RNA and glycogen (figure 114, p.286). They are now covered by a thin layer of keratin.

In the di-oestrus cultures there is complete separation in the highest concentrations and partial desquamation in the lowest. Thereafter the findings are similar to those at oestrus.

Cultures in medium 199 with vitamin A and oestradiol: In both oestrus and di-oestrus cultures the highest concentration (50 µg. of oestradiol and 100 µg. of vitamin A / ml.) produces degenerative changes within 24 hours. Thereafter the findings are essentially similar to those in medium 199 alone.

C. SUMMARY

The vaginal epithelium of adult mice forms keratin in culture medium 199. The epithelium has been removed at two stages in the cycle, oestrus and di-oestrus and thus provides a comparative study of the behaviour of non-keratinised epithelium with that in which there is preformed keratin. In both groups keratin is formed within 4 days in medium 199 alone. The addition of 5-0.05 µg./ml. of oestradiol accelerates the process in both groups. Testosterone (0.5-0.05 µg./ml.) progesterone (0.5-0.05 µg./ml.) corticosterone (10-0.01 µg./ml.) and vitamin A (10-1 µg./ml.) retard the process. The addition of either testosterone or vitamin A to cultures with oestradiol abolishes the acceleration produced by this steroid. Although the rate of keratin formation differs in the various groups there are no essential differences in the morphology or histochemistry of the various stages of the process. The transition from squamous epithelium to keratin is

associated with a diminution in the amount of glycogen and sulphydryl and the appearance of a reaction for disulphide. In all groups, but particularly in those associated with accelerated keratinisation the diminution in the amount of demonstrable sulphydryl is less than that found in normal or abnormal human skin or in the experimental lesions on the chick membrane. The amount of demonstrable disulphide in the keratin layer is also lower in amount than in the keratin layer in these sites.

DISCUSSION

The fundamental purpose of tissue culture is the maintenance of tissues alive outside the body. The various methods and their uses have been admirably summarised by Fell (1953). While she correctly distinguishes between "organ" and "tissue" culture it is customary and often more convenient to use the terms interchangeably. Thus in the present study the cultures fall into two distinct groups (i) Cell Culture in which interest is directed towards the "unorganised" outgrowth of cells from a tissue explant and (ii) Organ Culture where interest is centred upon the behaviour of the tissue fragment which may show a certain amount of progressive differentiation.

The technique of cell culture stems from the work of Harrison (1907) who studied the development of embryonic nerve fibres in clotted frog lymph. Although the earlier work with this technique was concerned with morphology its subsequent development has been centred in the fields of virology and cell metabolism. In general, its value in the study of cellular morphology has proved to be limited.

In the present study this limitation has been confirmed. The findings with the outgrowths from squamous epithelium confirm the statement of Fawcett (1955) that "notwithstanding the multiplicity of cell types in vivo and the complexity of their

organisation, tissues, whatever their source give rise *in vitro* to a very limited number of cells, shapes and organisational patterns". Most of the outgrowths show a reticular pattern of cells whose morphology is typical of fibroblasts. Less frequently there is a solid sheet of epithelial-like cells. The morphology of these cells, however, rapidly alters on subculture and they are soon indistinguishable from fibroblasts. Earlier reports of the establishment of permanent cell lines from human skin are scanty, (Allgower *et al.*, 1952; Perry *et al.*, 1957). The latter authors underline the position when they state "criteria which will conclusively distinguish this cell type from other cell types have not yet been established".

In addition, cells may alter in culture as regards their biological behaviour. Thus it has been shown by Chang (1957) that strains can arise from the same clone which have differing nutritional requirements. Similarly starting with normal connective tissue cells Gey (1956) and Sanford (1958) have demonstrated the development of malignant strains which produce metastasising fibrosarcomata when reinoculated into animals. The position has been summarised by Eagle (1958) who states "when cells are put into culture there is a loss of functional stability. Selection is made by the nature of the technique for the cell capable of rapid growth. This rapid growth is associated with some loss of

function". There may be two possible explanations of this. The more specialised cell types may die out under the abnormal conditions of culture or, on the other hand, the cells may lose their specific character and revert to pluripotential cells.

This reversion is usually referred to as dedifferentiation. This topic has been extensively reviewed earlier by Bloom (1937). At the outset he points out the difficulty in defining the term "differentiation" and that most definitions convey the idea of a progressive specialisation of form and function of cells of a developing organism but disregard their potentialities for further development. He also suggests that it is important to distinguish changes which may be only a temporary reaction of the cell to new stimuli and do not involve any change in potential function. It is to these reversible changes that Weiss (1953) has applied the term "modulation". Thus according to both these authorities a satisfactory definition of dedifferentiation must suggest that a cell shows some loss of actual function and an increase in potential functions. In the present series of cultures there appears to be selection of the cells capable of rapid growth. These cells are either fibroblast in type from the outset or even if initially epithelial, rapidly assume fibroblast-like morphology. Thus, the appearances are rapidly those associated with dedifferentiated cells in culture. They show no morphological or histochemical evidence of keratin formation. Even when the cells degenerate

they do so in a manner indistinguishable from other cell types in culture. In particular, there is no evidence of keratohyalin. Thus, while these cells do show the loss in culture of a specific function there is no evidence whether they have acquired any other potential function.

It is evident therefore that this particular method of culture although of intrinsic interest, is not suitable for a study of the possible function of an integrated piece of tissue, such as squamous epithelium and its development of keratin.

The technique of organ culture by contrast has proved of considerable value in the present study. It will be recalled that interest in this type of culture is centred on the integrated development of the explanted tissue rather than the outgrowth of cells from it. In general, this development is sought by providing conditions which permit slower growth and may resemble more closely those which are present in vivo. Several features are of importance in this connection. Thus the basis of this type of culture is the provision of a mechanical support which allows the ready access to the tissue of an adequate supply of oxygen and a fluid medium which is sub-optimal for rapid cell growth.

The combination of the lens paper and sponge, used as a support in the present study, has proved particularly suitable for use with the thin flat tissues studied. It also avoids the

disadvantage of the clot technique which, while used as a support, also acts as a nutrient medium of unknown chemical composition. This support may introduce another factor in that the normal relationship of the cells to each other is not unduly disturbed either by tissue distortion or by active cell migration. The inter-relationship of cells and their possible inter-dependence on each other for purposes of differentiation interested the early workers in this field. Drew (1922, 1923) studied plasma clot cultures of tumours and other tissues and concluded that the presence of connective tissue cells was necessary for epithelial differentiation. In particular he referred to keratinisation in the presence of fibroblasts. Fischer (1924) produced keratin from cultures of chick iris in the absence of fibroblasts. He concluded that their differentiating influence was not specific and resulted from lack of oxygen and nourishment.

The findings in the present study suggest that he is correct. The possible effects of the gas phase in skin cultures has been studied by Medawar (1947, 1948). He showed that rabbit skin can survive under anaerobic conditions for one week but neither cell movement or division take place. More recently Trowell (1959) emphasised the importance of the gas phase in the culture of many types of tissues but stated that foetal rat skin grows equally well in 5% CO2 in either air or oxygen. Thus, the oxygen

requirements of skin would appear to be less critical than those of other tissues. This is probably a reflection of the relatively low rate of metabolism of squamous epithelium. It is considered that the present method permits ready gaseous exchange at the gas fluid interface. As the capacity of the chamber was designed to contain an adequate supply of oxygen for the maximum number of cultures for a period of 48 hours it is considered that with twice daily flushing with 5% CO2 in oxygen the supply of oxygen to the cultured tissues is adequate.

It is much more probable that the most important factor, influencing the process of differentiation in this type of culture concerns the medium. The various synthetic media in current use have been evaluated recently by Paul (1960). He describes Medium 199 as elaborate and suggests that it may have unnecessary ingredients. However, while this medium may be adequate for the maintenance of established cell lines, it must be supplemented by serum or embryo extracts for the establishment and maintenance of recently isolated cell lines. In the present study the addition of these supplements (Tables XVII and XVIII, pp. 191, 192) produces a luxuriant outgrowth of cells which are morphologically similar to those already described in the cell cultures. Without additives the medium does not support vigorous cell growth. It does however permit the differentiation of epithelial tissues with

the production of keratin.

Considering the extensive literature on various aspects of cell culture, the field of organ culture has been relatively neglected. Previous work on keratinisation *in vitro* has been mainly concerned with the process in the infantile mouse vagina and the effects of oestrogens upon it.

Thus, the only comparable studies on foetal skin are restricted to animal tissues, notably the chick. Apart from the now classical work of Fell and Mellanby (1953), which will be considered later, two studies are of some relevance to the present investigation. Miszurski (1937) subjected plasma clot cultures of chick skin to conditions of impaired nutrition and oxygenation. He concluded that keratinisation represented the final stage in differentiation rather than a degenerative process. He further suggested that his findings disproved the theory of antagonism between proliferation and differentiation as there was no acceleration of keratin formation in conditions which cause a decline in proliferation. Litvac (1939) studied the keratinisation of chick skin in plasma clot cultures with the sodium nitroprusside reaction for cystine. She noted that this was most strongly positive in the keratinising zone without any reaction in the peripheral germinative zone or in the fully formed keratin layer.

With adult human skin the only comparable study is that of Matoltsy and Sinesi (1957) who followed keratin formation in plasma clot cultures by the DDD method. The pre-existing keratin was removed by Scotch tape. They concluded that keratin formation takes place when proliferation is inhibited in culture and the squamous cells are simultaneously stimulated to differentiate by the removal of keratin.

On the whole, the present study confirms and extends these findings. It will be recalled that the mode of keratin formation in vitro is similar in all the tissues studied. Even in those tissues (foetal human skin, 12 day foetal mouse skin and infantile mouse vagina) where the epithelium is cuboidal, in culture it rapidly becomes squamous in type. Thereafter it behaves in an essentially similar manner to the tissues in which the epithelium is initially squamous.

The histochemistry of the process is again similar in all the tissues studied and in keeping with that found in the process in vivo. Thus, there is the inverse relationship between the degree of keratinisation and the amount of demonstrable glycogen. The assumption of squamous morphology is accompanied by the appearance of a reaction for sulphydryl. There is considerable increase in this reaction just before the squamous cells become keratinised,

while keratin formation is manifest by the appearance of a reaction for disulphide. While the morphology and histochemistry suggest a more rapid and probably less complete form of keratinisation than that which occurs in vivo, the basic process is essentially similar.

The question as to whether keratinisation represents a differentiation or degeneration as raised by Miszurski (1937) has been admirably discussed by Pinkus (1954). He points out that the end-product of a process of differentiation, although dead, may still be able to fulfil a highly specialised function. In particular, he cites red blood cells. The findings in the present study suggest that any controversy may be rather pointless as both processes are exhibited during keratin formation. Thus, in culture, the squamous cell precursors, i.e., the foetal cuboidal cells and the adult basal cells proliferate. For a limited period they and their successors are changed into apparently mature squamous cells, i.e., they are differentiated in that they have only one remaining function - the formation of keratin. Their final devitalisation to form acellular keratin however is a degenerative phenomenon.

In the present study it was not found necessary to stimulate the existing squamous cells to differentiate by removing the keratin layer as did Matoltsy and Sinesi (1957). The findings

suggest that once the cells have become squamous in type, they must inevitably proceed towards some degree of keratinisation as the process of keratinisation is the way in which these highly differentiated cells must degenerate. Again, the present findings are not in keeping with the suggestion of Miszurski (1937) that there is no antagonism between proliferation and differentiation. It may be that the existing squamous cells differentiate by what Carrel (1924) called "residual growth energy". They are however replaced by cells derived from relatively undifferentiated epithelial cells. This replacement by typical squamous cells lasts only for a limited period of time. As the germinal cells become adjusted to the suboptimal conditions of growth, they begin to grow more rapidly, assume less distinctive morphology and no longer differentiate to become mature squamous cells. Eventually, even under these conditions of culture, which are designed to restrict growth, the cells actively proliferate. As assessed by morphology, they progress only partly towards squamous differentiation and show loss of normal function in that they do not produce keratin. While they are dedifferentiated in this respect, there is no evidence whether or not they have acquired any other potential function.

An attempt to determine whether these cells have the capacity to develop in other ways has been made by the addition to the

medium of substances known to influence the process of keratinisation *in vivo*. They may be divided conveniently into two groups (a) those associated with inhibition of keratin formation, notably vitamin A and, (b) those associated with increased keratin formation, including oestradiol and other steroids.

The antikeratinising action of vitamin A *in vivo* is well established (Mori, 1922 a & b, 1923; Wolbach & Howe, 1925, 1928). While the *in vitro* studies are relatively scanty, the findings of Fell and Mellanby (1953) are of considerable interest. They found that if 10-30 I.U. of vitamin A was added to cultures of 7 day chick skin keratin was not formed and a mucoid secretory ciliated type of epithelium developed. Their findings were substantiated by Weiss and James (1955) who exposed fragments of chick skin for brief periods to a medium containing 60 µg./ml. at 2 day intervals. They suggested that the vitamin acts as an inductive agent which has switched the cellular mechanism of differentiation into another course. They state that this is an example of modulation. In the present study the vitamin produces less striking results. Thus, in the tissues whose epithelium is already squamous in type, there is a slowing of the conversion of the existing squamous cells into keratin. In the undifferentiated tissues there is also slowing of the conversion of the precursors into squamous cells. The effect,

however, lasts for a comparatively short period of time before the cells become squamous and eventually keratinised. The findings suggest that in all the various tissues studied, once the cells have developed from the germinal cells they are irreversibly differentiated towards squamous cells which inevitably keratinise. Even the germinal cells which are presumably less well defined as to their future line of development show no evidence of deviating from this form of differentiation.

It is possible that, although the dosages employed are greatly in excess of normal blood levels, the activity of the vitamin declines in culture. To minimise this possibility the medium in these cultures was changed at 12 hourly intervals. Again, the effects produced by Weiss and James (1955) were obtained by the exposure of skin to the vitamin for only 30 minutes at 2 day intervals. The mucous metaplasia produced by Fell and Mellanby (1953) often took 9 days to develop and few of the tissues in the present series have been followed for that length of time. This would not appear to be significant as, even in the tissue which is most reactive (human foetal skin), some of the cells are squamous within 4 days. In the tissues studied by Fell and Mellanby (1953) there was no evidence of squamous differentiation at any stage. These authors mention briefly that preliminary studies showed that mouse skin was much less reactive than that of the chick. There may well be a species

variation in sensitivity as the blood levels and vitamin requirements of various animals vary enormously. It is also possible that if younger embryos had been used in the present study the skin might have proved to be more reactive. This would appear to be unlikely as in the foetal tissues at least, the germinal cells might reasonably be expected to be multipotential.

It is of considerable interest that, although more toxic, squalene produced essentially similar findings in the lowest concentrations. Its use in the present study was prompted by the suggestion by Flesch (1952, 1953), on the basis of clinical and experimental animal studies, that it might have an anti-keratinising effect. Although he draws no firm conclusions, he suggests that the effect of vitamin A is probably non-specific. It is also of interest that in discussing their findings Fell and Mellanby (1953) state that the vitamin may not be directly responsible for the mucous metaplasia but is merely suppressing keratinisation. Again this is in keeping with the present findings. It is tempting to speculate as to the mode of action of these substances. However, from the present findings one can only agree with the statement of Moore (1957) in his authoritative textbook that "at present any conclusion which would narrow down our theories as to the mode of action of vitamin A would seem unjustified". It remains that the present study shows that in the various

epithelial tissues removed from any possible inhibiting mechanism of the intact organism, the addition of vitamin A produces no deviation from normal squamous development with subsequent transformation of the squamous cells into keratin. The morphology and histochemistry of the process suggest that it is essentially similar to that in the other sites, considered earlier.

As discussed in part IV oestrogens are known to stimulate the keratinisation of vaginal epithelium in vivo. While there have been more studies in vitro with vaginal epithelium than with skin, those relevant to the present study have utilised only infantile tissues. The effect of oestrogens added to these cultures of cuboidal epithelium has produced conflicting results. Thus Emmens and Ludford (1940) failed to induce keratinisation in rat vaginal epithelium in plasma clot cultures which contained oestradiol. Dux (1941) found that, while similar tissues did produce keratin, oestrogens did not directly affect the process although in cultures in serum from rats at oestrus there was an acceleration. More recently Hardy et al. (1953), Biggers et al. (1956) and Kahn (1954) produced keratin from immature epithelium from mice and rats. Later Kahn (1959) found that the addition of oestradiol to a synthetic medium accelerated the process. The most recent study is that of Martin (1959) who used both biological and synthetic media and found that while keratin was formed, the

addition of oestrone, cortisone, testosterone or dimethyl stilboestrol did not affect the process. At the outset, a possible explanation of the discrepancy of the findings in the present study with those of Martin (1959) may be that he followed his cultures for only 3 days and keratin formation appears to have occurred more rapidly under the conditions of his experiments. In the present study the addition of oestradiol accelerates the rate of keratin formation in epithelium which is already squamous in type. It also accelerates the rate of conversion of foetal epithelial cells to squamous cells which also keratinise rapidly. Although it is accelerated, and the histochemical findings are consistent with the formation of keratin being less complete, the process is essentially similar to that which occurs in vivo.

All the tissues studied react to the addition of this steroid although the most sensitive are the vaginal tissues. As discussed in part IV this is as might be expected, although skin is also an oestrogen sensitive tissue (Bullough H.F., 1942-44). Burrows (1949) pointed out that the capacity to respond to oestrogens and the degree of response are innate cellular characteristics. These characteristics are retained when the tissues are transferred to other parts of the body (Raynaud, 1930). As shown in the present study they are also retained for a limited time in vitro. However, after a short

period, in this environment, the germinal cells no longer differentiate into squamous cells. They assume the morphology of much more rapidly proliferating cells and thereafter show no evidence of differentiation as found in the cultures in medium alone.

It is possible that the addition of oestrogen supplies an ingredient which is lacking in the medium used. The effects produced by the other steroids however, renders this possibility unlikely. Thus, while keratin formation is also accelerated by dehydroisoandrosterone, the addition of testosterone, progesterone and corticosterone retard the process. While the similarity of effect produced by an oestrogen and an androgen are puzzling they are, nevertheless, in keeping with *in vivo* findings. The most relevant of these to the present study showed that the administration of different androgens produced different effects in hypophysectomised rats. Thus, while many androgens were inactive the administration of testosterone produced a "mucified" vaginal epithelium while 5-androstene 3:17 dione produced keratin (Huggins *et al.* 1954). Again although the precise mode of action of steroids is unknown their effects on vaginal epithelium is generally well characterised. The interaction and possible antagonism are less well established and the literature on this topic is confusing and largely contradictory. Thus, from a study

of the previous literature and carefully controlled animal experiments Edgren (1959) concludes that "it is difficult to predict how a given steroid will react and the same steroid may be either synergistic or antagonistic". It is of interest therefore, that the present findings show that corticosterone, testosterone and progesterone all abolish the acceleration produced by oestradiol. Moreover their effects are indistinguishable from those of vitamin A. The antagonism of these substances suggests there be some specificity of action although there is no evidence that the basic process of keratinisation is altered in any way. Thus, while the findings do not offer any evidence as to the mode of action of these substances, they are still consistent with the suggestion of Bullough (W.S. 1953) and Martin and Claringbold (1958) that the action of oestrogens is purely mitogenic and it is equally possible that the inhibitory action of the other steroids is mediated through their effects on the mitotic activity of the germinal cells.

C O N C L U S I O N

The findings and their possible interpretation have been already discussed at the end of the appropriate parts. It is now convenient to consider very briefly what has emerged from this fairly wide consideration of the process of keratinisation and to suggest possible future developments. It has been shown that the histochemistry of the process in normal human skin is consistent with the theory that the conversion of sulphydryl to disulphide is the essential step in the transformation of the epidermal cells into acellular keratin. A similar process has also been shown in human skin lesions which are associated with abnormal forms of keratinisation and in the in vivo studies in the developing egg and in LAF 1 mice. The histochemical methods available at present are still relatively crude. As they become more refined and possibly used in conjunction with the recently available enzyme keratinase, it may be possible to explore the pre-keratins further. An essentially similar process has been demonstrated in vitro in both human and animal tissues. It has been shown that adult and foetal epithelium can form keratin in an artificial culture medium of known chemical composition. In this environment which is unfavourable for active cell growth the formation of keratin takes place in an indistinguishable manner from that in vivo. It may be that the tissues studied, even the foetal tissues have already only the potential to develop into squamous epithelium. The behaviour of human foetal skin from

different aged embryos therefore, would be of considerable interest. In the present study it has not been shown possible to alter the process of keratin formation in any type of epithelium. The various substances added, in general, have the effect of altering the rate at which the process takes place. The effect of a wider range of substances, particularly the vitamin analogues and a wider range of steroids, may well prove of value particularly in the mechanism of interaction of the various steroids. It may be that the tissues studied have only the potential for squamous development and subsequent keratin formation. Therefore, a similar investigation into the behaviour in culture of other types of epithelium might prove to be of considerable interest. Much remains uncertain about the process of keratin formation and further in vitro studies may, with a combination of other techniques, such as electron microscopy or autoradiography, help in its elucidation.

ACKNOWLEDGEMENT

I wish to thank Professor T. Symington, Drs. J. Furth, J.C.J. Ives and F.O. McCallum for help and advice at various stages of this work. Professor Symington has also provided invaluable criticism during the preparation of the manuscript.

Some of the material described in Parts II, III and IV has already been published jointly (Pullar and Cochrane, 1957; Ghera et al., 1960). Assistance has been provided by many past and present members of the technical staff of the Royal Infirmary. I am particularly indebted to Mr. D.F. Hay, Mr. J. Hendry and Miss Janet Irvine. The photographs are the work of Mr. T. Parker. Considerable secretarial help has been given by Miss Margaret Stuart and Miss Pauline Fox.

MORPHOLOGICAL AND EXPERIMENTAL STUDIES ON KERATINISATION

Thesis submitted for the degree of Doctor of

Medicine of the University of Glasgow

By

PETER PULLAR, M.B., Ch.B.

VOLUME II (APPENDIX)

TABLE I

Composition of Representative Keratins

Keratin	Wool	Hair	Horn	Quill	Feather	Epidermis
Source	Sheep	Human	Cattle	Porcupine	Chicken	Human
Type	Hard	Hard	Hard	Hard	Hard	Soft
Structure	α	α	α	α	β	α
Component	Grams of component from 100 g. of dry keratin					
Nitrogen, total	16.2-16.9	15.5-16.9	14.8-16.9	15.8	15.0-16.2	14.2-15.5
Amide nitrogen	1.10-1.37	1.17	1.14		1.09	1.16
Sulphur	3.0-4.0	5.00-5.24	3.77-3.9	3.0	2.9	1.9
Amino acids						
With hydrocarbon side chains						
Glycine	5.2-6.5	4.1-4.2	9.6	5.7	7.2	6.0-13.8
Alanine	3.4-4.4	2.8	2.5		5.4	
Valine	5.0-5.9	5.5-5.9	5.3-5.5		8.3-8.8	4.2-5.6
Leucine	7.6-8.1	6.4-8.3	7.6-8.3		7.4-8.0	8.3
Isoleucine	3.1-4.5	4.7-4.8	4.3-4.8		5.3-6.0	6.8
Phenylalanine	3.4-4.0	2.4-3.6	3.2-4.0	3.6	4.7-5.3	2.8
Proline	5.3-8.1	4.3-9.6	8.2		8.8-10.0	3.2
Hydroxy						
Serine	7.2-9.5	7.4-10.6	6.8	6.1-6.2	10.2-14	16.5
Threonine	6.6-6.7	7.0-8.5	6.1	3.9-5.4	4.4-4.8	3.4
Tyrosine	4.0-6.4	2.2-3.0	3.7-5.6	3.3	2.0-2.2	3.4-5.7
Acid (free and as amide)						
Aspartic acid	6.4-7.3	3.9-7.7	7.7-7.9	8.7	5.8-7.5	6.4-8.1
Glutamic acid	13.1-16.0	13.6-14.2	13.8	17.6	9.0-9.7	9.1-15.4
Basic						
Arginine	9.2-10.6	8.9-10.8	6.8-10.7	7.6-8.0	6.5-7.5	5.9-11.7
Lysine	2.8-3.3	1.9-3.1	2.4-3.6	2.6	1.0-1.7	3.1-6.9
Hydroxylysine	0.2	0				
Histidine	0.7-1.1	0.6-1.2	0.6-1.0	0.6	0.3-0.7	0.6-1.8
Heterocyclic						
Tryptophan	1.8-2.1	0.4-1.3	0.7-1.4	0.9	0.7	0.5-1.8
Sulphur-containing						
Cystine	11.0-13.7	16.6-18.0	10.5-15.7	8.0-9.5	6.8-8.2	2.3-3.8
Methionine	0.5-0.7	0.7-1.0	0.5-2.2	0.8	0.4-0.5	1.0-2.5
Cysteine	0.4	0.5-0.8	0.8-1.6		0.4	

TABLE II

AMINO ACID COMPOSITION IN PERCENT OF AMINO NITROGEN

Source	Arginine	Histidine	Lysine	Tyrosine	Tryptophane	Phenylalanine	Cystine	Methionine	Serine	Threonine	Leucine	Isoleucine	Valine	Glutamic Acid	Aspartic Acid	Glycine	Alanine	Proline	Hydroxy-proline
Heat separated epidermis	3.6	2.4	5.8	3.4	2.2	5.0	4.8	1.9	9.7	5.3	9.0	3.7	6.6	14.1	5.5	7.8	6.5	4.9	4.5
Normal (Callus)	4.3	2.9	7.9	3.7	2.5	7.1	9.1	2.0	8.3	6.0	10.2	2.1	6.7	13.2	4.7	3.7	6.9	6.6	None

TABLE III

The number of patients examined

and the clinical grouping of the warts

No. of patients	Hyperkeratotic	Dyskeratotic	Mixed Type	Periungual	Filiform	Plane
164	104	33	3	9	13	2

TABLE IV

Clinical features of warts

Type of wart	Age		Number		Situation			Duration				History of Contact	
	8–20 yrs.	20 yrs.+	1–6	7+	Palms	Dorsa	Other	1–8 wks.	2–6 mths.	½–2 yrs.	2+ yrs.	Certain	Doubtful
Hypkereratotic	66	38	46	58	4	74	26	2	15	46	41	3	9
Dyskeratotic	11	22	31	2	26	3	4	15	11	4	3	16	5
Both	3	–	3	–	3	–	–	2	1	–	–	–	3
	–	–	–	3	–	3	–	–	1	2	–	–	–
Periungual	5	4	6	3	–	–	9	–	2	3	4	–	2
Filiform	5	8	6	7	–	–	13	1	2	9	–	–	3
Plane	2	–	–	2	–	2	–	–	–	1	1	–	–

TABLE V

Summary of main clinical differences

between hyperkeratotic and dyskeratotic warts

	Hyperkeratotic	Dyskeratotic
Site	various	palmar
Situation	superficial	deep
Number	multiple	single
Pain	seldom	always
Duration	long	short
Recurrence	often	never
Source	seldom	often

TABLE VI

Material inoculated in fertile eggs

Material	No. of lesions inoculated*				
	CAM.	Amnion	Yolk Sac	Brain	Skin Graft
Hyperkeratotic wart	35	5	4	3	28
Dyskeratotic wart	4	2	1	3	6
CAM previously inoculated with hyperkeratotic lesions	7	-	-	-	-
CAM previously inoculated with dyskeratotic lesions	3	-	-	-	-

* 3 eggs were inoculated with each lesion

TABLE VII

Grafts on CAM

	No.	No. Viable After 4 Days
Human Skin	83	75
Rat Embryo Skin	10	8
Squamous cell carcinoma	8	1
Keratoacanthoma	6	1
Simple Melanoma	4	1

TABLE VIII

Passage of adrenotrophic tumour (No. 20)

Passage No.	No. of Animals			
	Intact	Adrex.	Gonex.	Adrex. and Gonex.
20		7/7*(31)		
21	10/10 (30)			
22	6/6 (28)			
23	11/12 (30)			
24	5/6 (30)	5/5 (29)		
25		5/6 (30)	6/6 (30)	5/6 (34)
26	5/6 (26)	4/4 (28)		
27	4/4 (29)	10/10 (28)		
28	8/8 (28)			
29	6/6 (26)			

* Number of animals with tumours over number grafted. Average latency (i.e., time until the tumour became palpable) in days is given in parentheses.

TABLE IX

Passage of adrenotrophic tumour (No. 2)

Passage No.	No. of Animals
12	5/5 (95)*
13	5/5 (80)
14	0/5**

* Number of animals with tumours over the number injected. The latency is given in parentheses.

** In progress.

TABLE X

Operative treatment of

LAF1 mice bearing adrenotrophic tumour No. 20

Passage No.	Intact	"Adrex."	"Gonex."	"Adrex. and Gonex."
24	5	5		
25		5	6	5
26	5	4		
27	4	10		
Total	14	24	6	5

TABLE XI

Vaginal smears of adrenotrophic tumour bearing LAF1 mice (strain 20)

Day	Controls	At.T.	At.T. + Adrex.	At.T. + Gonex.	At.T. + Adrex. Gonex
1	P	P	P	P	P
2	O	P	O	P	P
3	O	P	O	P	P
4	M	P/O	M	P	P
5	D	D	M	D	P
6	D	D	D	P	P
7	P	P	P	P	P
8	O	P	O	P	P
9	O	P	O	D	D
10	M	P	M	D	D
11	D	D	M	D	D
12	D	P	D	P	P
13	P	P	P	P	P
14	O	P	O	P	P
15	O	P	O	D	P
16	M	P/O	M	D	P
17	D	D	M	P	P
18	P	D	D	P	P
19	O	P	P	P	P
20	O	P	O	P	P
21	M	P	O	D	P

O = oestrus
At.T. = adrenotrophic tumour
P = pro-oestrus
M = meta-oestrus
D = di-oestrus

TABLE XII

Vaginal smears of adrenotrophic tumour bearing LAF1 mice (strain 2)

Day		Tumour Size
1	O/M	Not palpable
2	D	
3	D	
4	D	
5	P	
6	P	
7	P	
8	P	
9	P	
10	P	
11	P	
12	P	
13	P	
14	P	
15	P	←Palpable
16	P	
17	P	
18	P	
19	P	
20	P	
21	P	5 mm. in diameter (at autopsy)

TABLE XIII

Passages of thyrotrophic tumour in LAF1 mice

Passage	Intact	ATHYROID
3	4/6** (438)	6/6 (400)
4	1/6* (402)	5/6 (389)

* In progress.

** Number of animals with tumours over the number injected.

Latency in days in parentheses.

TABLE XIV

Media used in Plasma Clot Cultures

	Total No. of Cultures	Growth		
		Good	Poor	None
1. Medium 199 80% Human Serum 20%	33	8	7	18
2. 0.5% Lactalbumin hydrolysate in Earle's salt solution 80% Human Serum 20%	15	1	1	13
3. 0.5% Lactalbumin hydrolysate in Earle's salt solution 65% Human Serum 30% Chick Embryo extract 5%	10	1	2	7
Total	58	10	10	38

TABLE XV

Plasma clot cultures on coverslips

	No. of cultures	Growth		
		Good	Poor	None
Skin				
Adult Human	25	2	6	17
Foetal Mouse	3	2	1	0
Foetal Rat	3	2	1	0
Foetal Chick	5	4	1	0
Total	36	10	9	17
Other Lesions				
Simple Melanoma	12	0	0	12
Basal cell carcinoma	7	0	1	6
"Basal cell" papilloma	2	0	0	2
Squamous cell carcinoma	1	0	0	1
Total	22	0	1	21

TABLE XVI

Plasma clot cultures in watch glasses

	No. of Cultures	Growth		
		Good	Poor	None
Skin				
Human	10	1	5	4
Chick	10	7	2	1
Other Lesions				
Simple Melanoma	2	0	0	2
Basal Cell Carcinoma	2	0	0	2
Keratoacanthoma	1	0	0	1

TABLE XVII

Media used in preliminary organ cultures

		No. of Cultures
Eagle's Medium Mouse or Human Serum Chick Embryo Extract	60% 20% 20%	80
S.M. 199 Mouse or Human Serum	80% 20%	30
S.M. 199 Mouse or Human Serum Chick Embryo Extract	60% 20% 20%	30
S.M. 199 Bovine Amniotic Fluid	50% 50%	90
S.M. 199 Alone		20
S.M. 858 Bovine Amniotic Fluid	50% 50%	50
S.M. 858 Alone		40

TABLE XVIII

Preliminary organ cultures of mouse tissues

Tissue		No. of Cultures
Skin	Foetal	30
	Infantile	30
Vagina	Foetal	12
	Infantile	12
Adrenal	Foetal	30
	Infantile	45
Thyroid	Foetal	10
	Infantile	90
Other Tissues: (Pituitary, thymus, prostate, and various mouse tumours)		81

TABLE XIX

MEDIA

SYNTHETIC MEDIUM 199 (0477)

Ingredients per litre

l-Arginine	70 mg.	Pantothenate	0.01 mg.
l-Histidine	20 mg.	Biotin	0.01 mg.
l-Lysine	70 mg.	Folic Acid	0.01 mg.
l-Tyrosine	40 mg.	Choline	0.5 mg.
dl-Tryptophane	20 mg.	Inositol	0.05 mg.
dl-Phenylalanine	50 mg.	p-Aminobenzoic Acid	0.05 mg.
l-Cystine	20 mg.	Vitamin A	0.1 mg.
dl-Methionine	30 mg.	Calciferol	0.1 mg.
dl-Serine	50 mg.	Menadione	0.01 mg.
dl-Threonine	60 mg.	a-Tocopherol Phosphate	0.01 mg.
dl-Leucine	120 mg.	Ascorbic Acid	0.05 mg.
dl-Isoleucine	40 mg.	Glutathione	0.05 mg.
dl-Valine	50 mg.	Cholesterol	0.2 mg.
dl-Glutamic Acid	150 mg.	Sodium Acetate	50 mg.
dl-Aspartic Acid	60 mg.	l-Glutamine	100 mg.
dl-Alanine	50 mg.	Adenosinetriphosphate	1 mg.
l-Proline	40 mg.	Adenylic Acid	0.2 mg.
l-Hydroxyproline	10 mg.	Iron (as Ferric Nitrate)	0.1 mg.
Glycine	50 mg.	Ribose	0.5 mg.
l-Cysteine	0.1 mg.	Desoxyribose	0.5 mg.
Adenine	10 mg.	Tween 80	0.005 mg.
Guanine	0.3 mg.	Sodium Chloride	8 g.
Xanthine	0.3 mg.	Potassium Chloride	0.4 g.
Hypoxanthine	0.3 mg.	Calcium Chloride	0.14 g.
Thymine	0.3 mg.	Magnesium Sulphate	0.2 g.
Uracil	0.3 mg.	Disodium Phosphate	0.06 g.
Thiamine	0.01 mg.	Monopotassium Phosphate	0.06 g.
Riboflavin	0.01 mg.	Sodium Bicarbonate	0.35 g.
Pyridoxine	0.025 mg.	Bacto - Dextrose	1 g.
Pyridoxal	0.025 mg.	Bacto - Phenol Red	0.02 g.
Niacin	0.025 mg.	Carbon Dioxide to pH	7.2
Niacinamide	0.025 mg.	Triple Distilled Water	1000 ml.

TABLE XX

Organ Culture of Squamous Epithelium

Total Number of Cultures

	Human		Mouse		
	Adult	Foetal	Adult	Foetal	Infantile
Skin	224	12	0	432	0
Vagina	0	0	228	0	276

TABLE XXI

Organ culture of human foetal skin

Added to Medium µg./ml.	No. of Cultures
Vitamin A Acetate. 500	4
Oestradiol. 50	4
Control	4

TABLE XXII

Organ culture of adult human skin

Added to Medium µg./ml.		No. of Cultures
Oestradiol	50	15
	5	15
	0.5	15
	0.05	15
Dehydroisoandrosterone	50	3
	5	3
	0.5	3
	0.05	3
Vitamin A	1000	18
	100	18
	10	18
	1	18
Squalene	1000	3
	100	3
	10	3
	1	3
Vitamin A and Oestradiol	50	
	50	9
	5	9
	0.5	9
	0.05	9
Control		32

TABLE XXIII

Organ culture of foetal mouse skin

Added to Medium µg./ml.		No. of Cultures	
		Keratinised	Non-Keratinised
Oestradiol	50	6	18
	5	6	18
	0.5	6	18
	0.05	6	18
Dehydroisoandrosterone	50	3	3
	5	3	3
	0.5	3	3
	0.05	3	3
Vitamin A	1000	9	21
	100	9	21
	10	9	21
	1	9	21
Squalene	1000	-	6
	100	-	6
	10	-	6
	1	-	6
Vitamin A and Oestradiol	500		
	50	9	21
	5	9	21
	0.5	9	21
	0.05	9	21
Control		12	36

TABLE XXIV

Organ culture of infantile mouse vagina

Added to Medium µg./ml.		No. of Cultures
Oestradiol	50	30
	5	30
	0.5	30
	0.05	30
Testosterone	50	9
	5	9
	0.5	9
	0.05	9
Corticosterone	10	6
	1	6
	0.1	6
	0.01	6
Vitamin A	1000	12
	100	12
	10	12
	1	12
Oestradiol and Testosterone	50	
	50	6
	5	6
	0.5	6
	0.05	6
Control		24

TABLE XXV

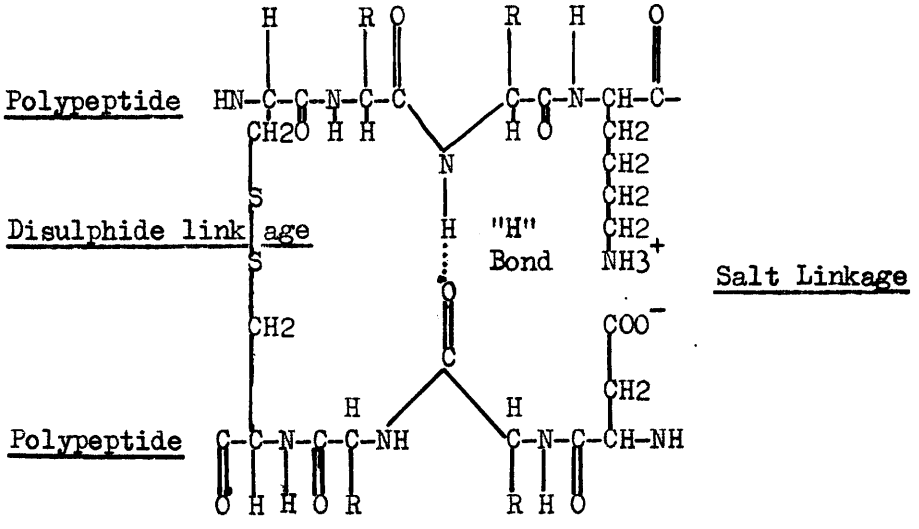
Organ culture of adult mouse vagina

Added to Medium µg./ml.		No. of Cultures	
		Oestrus	Di-oestrus
Oestradiol	50	5	10
	5	5	10
	0.5	5	10
	0.05	5	10
Progesterone	50	3	3
	5	3	3
	0.5	3	3
	0.05	3	3
Testosterone	50	3	3
	5	3	3
	0.5	3	3
	0.05	3	3
Corticosterone	10	3	3
	1	3	3
	0.1	3	3
	0.01	3	3
Vitamin A	1000	3	3
	100	3	3
	10	3	3
	1	3	3
Vitamin A and Oestrogen	100		
	50	3	3
	5	3	3
	0.5	3	3
	0.05	3	3
Oestradiol and Testosterone	50		
	50	3	3
	5	3	3
	0.5	3	3
	0.05	3	3
Control		12	12

FIGURE 1

THE THREE MAIN TYPES OF CROSS-LINKAGES

IN KERATIN MOLECULES



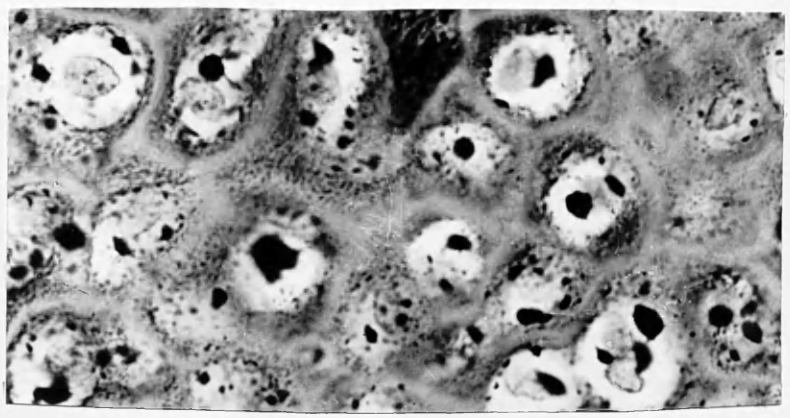
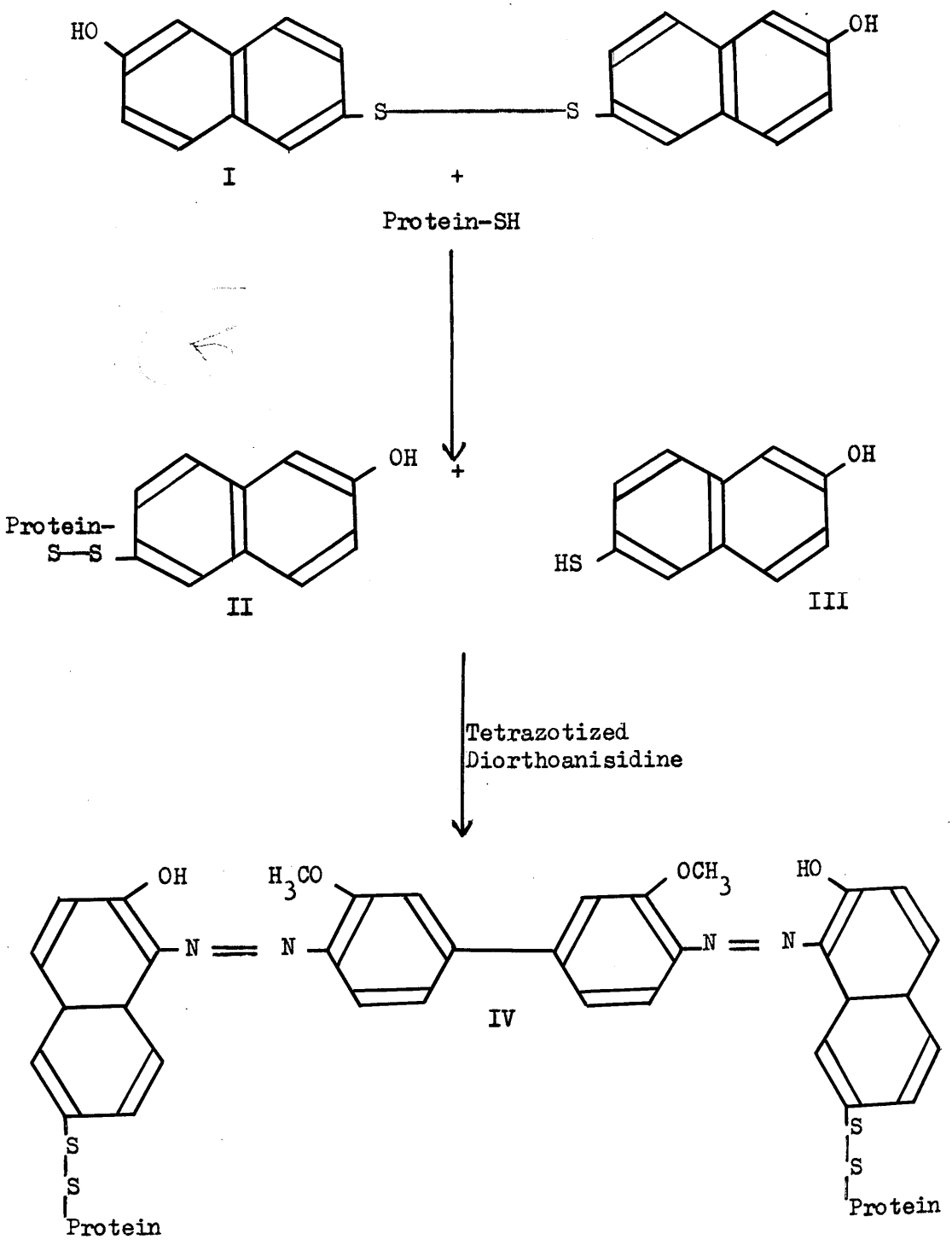


Fig. 2. Adult human skin; the transition zone between the squamous and keratin layers. Numerous keratohyalin granules are present.
Haematoxylin and eosin x 900.

FIGURE 3

THE DIHYDROXY-DINAPTHYL-DISULPHIDE METHOD

FOR SH AND SS



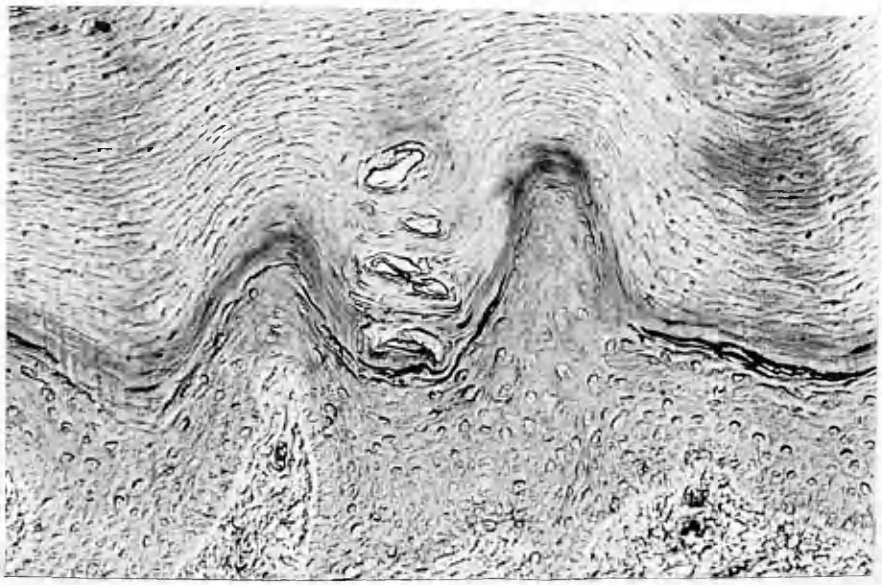


Fig. 4. Adult human skin; the distribution of SH groups. A zone of intensified staining is present at the junction of the squamous and keratin layers.
Dihydroxy-dinapthyl-disulphide x 280.

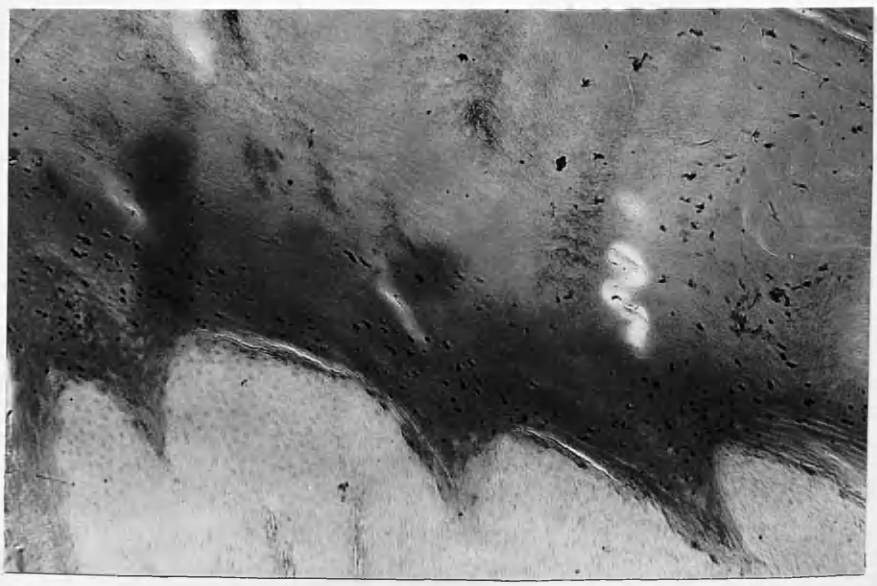


Fig. 5. Adult human skin; distribution of SS groups. Staining is confined to the keratin layer.
Dihydroxy-dinapthyl-disulphide x 90.

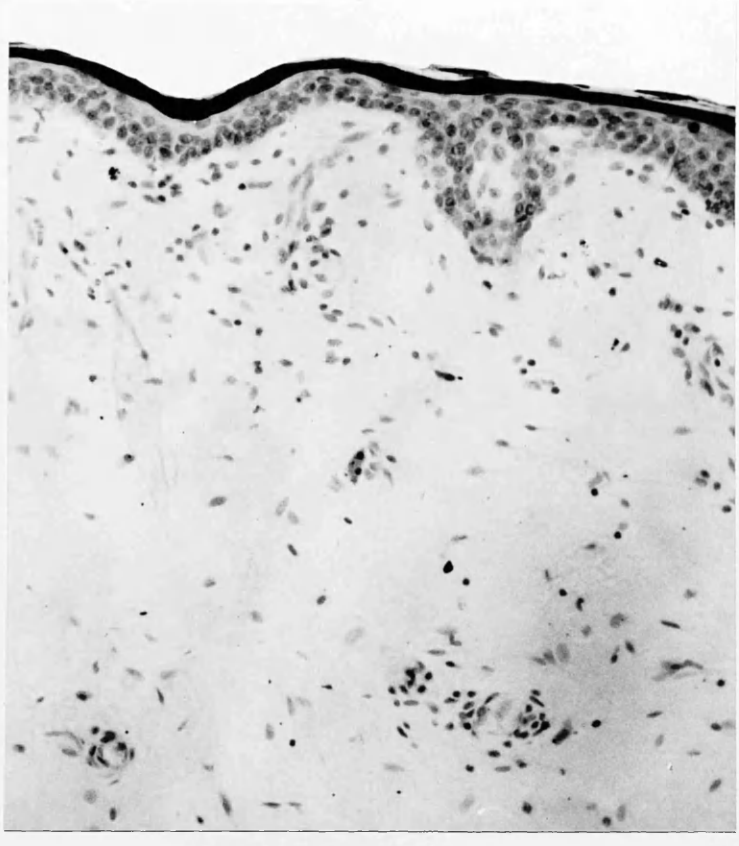


Fig. 6. Adult human skin; distribution of acid phosphatase. An intensely staining band is present in the granular layer.
Holt's method x 225.

Fig. 7. Hyperkeratotic papilloma; two superficial lesions on the dorsal surface.

Fig. 8. Dyskeratotic papilloma; a single deep seated lesion on the palmar surface.

Fig. 9. Hyperkeratotic papilloma; numerous vacuolated cells are present. Haematoxylin and eosin x 125.

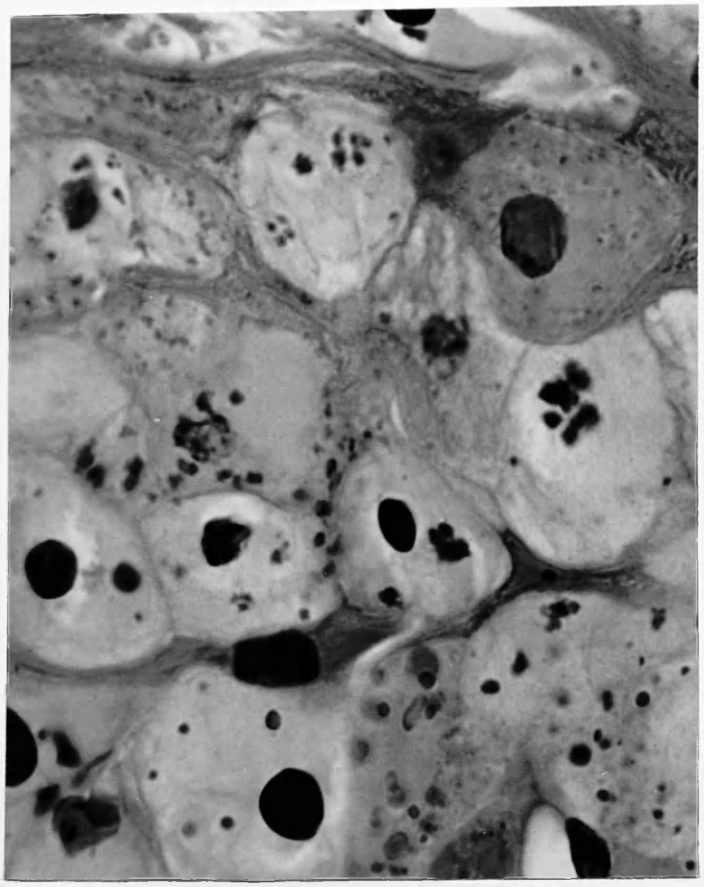


Fig. 10. Hyperkeratotic papilloma; pyknotic nuclei and nuclear debris in the granular layer. Haematoxylin and eosin x 950.

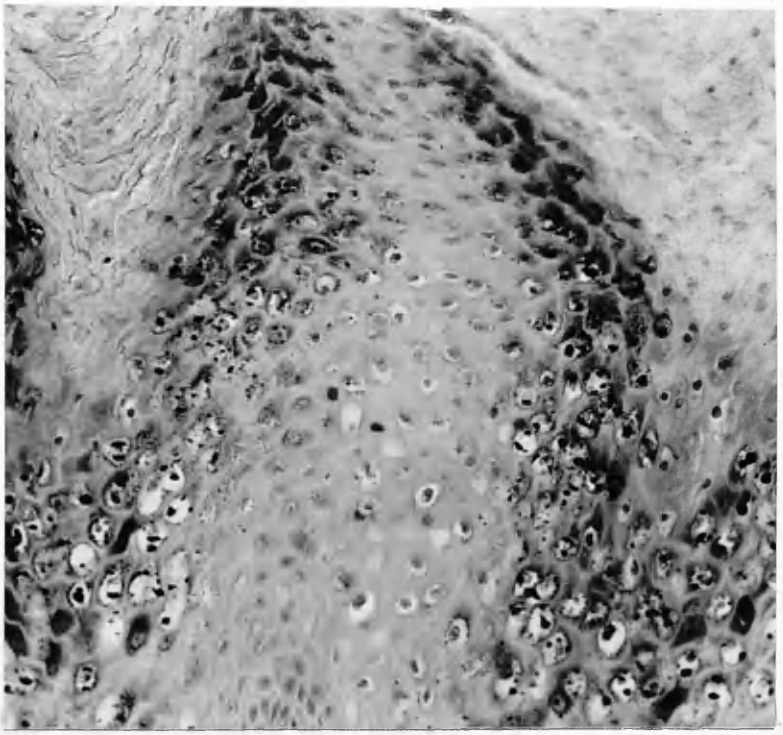


Fig. 11. Hyperkeratotic papilloma showing a prominent granular layer.
Haematoxylin and eosin x 225.

Fig. 12. Hyperkeratotic papilloma; parakeratotic nuclei in the keratin layer.
Haematoxylin and eosin x 90.

Fig. 13. Dyskeratotic papilloma; squamous cells are separated by masses of keratin. Numerous dyskeratotic cells are present.
Haematoxylin and eosin x 75.

Fig. 14. Dyskeratotic papilloma; an isolated group of dyskeratotic cells low in the squamous layer.
Haematoxylin and eosin x 225.

Fig. 15. Dyskeratotic papilloma; there are numerous dyskeratotic cells which contain vacuolated cytoplasmic masses and intranuclear bodies. Haematoxylin and eosin x 750.

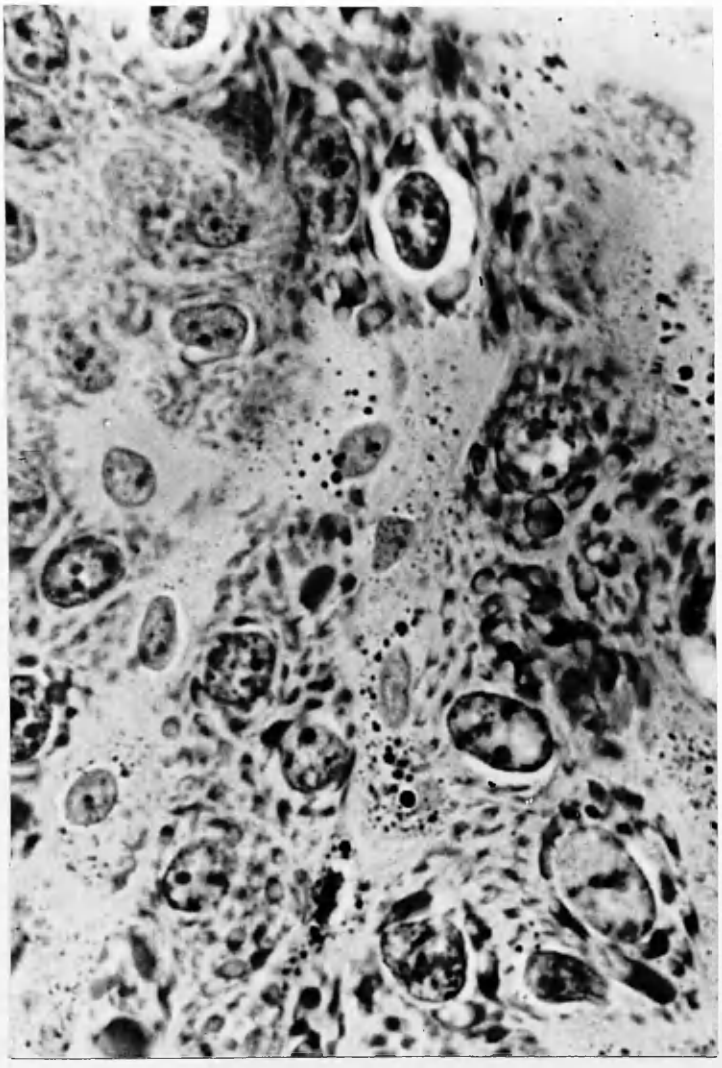


Fig. 16. Dyskeratotic papilloma; dyskeratotic cells in a partially keratinised area. Some nuclei are now swollen.
Haematoxylin and eosin x 950.

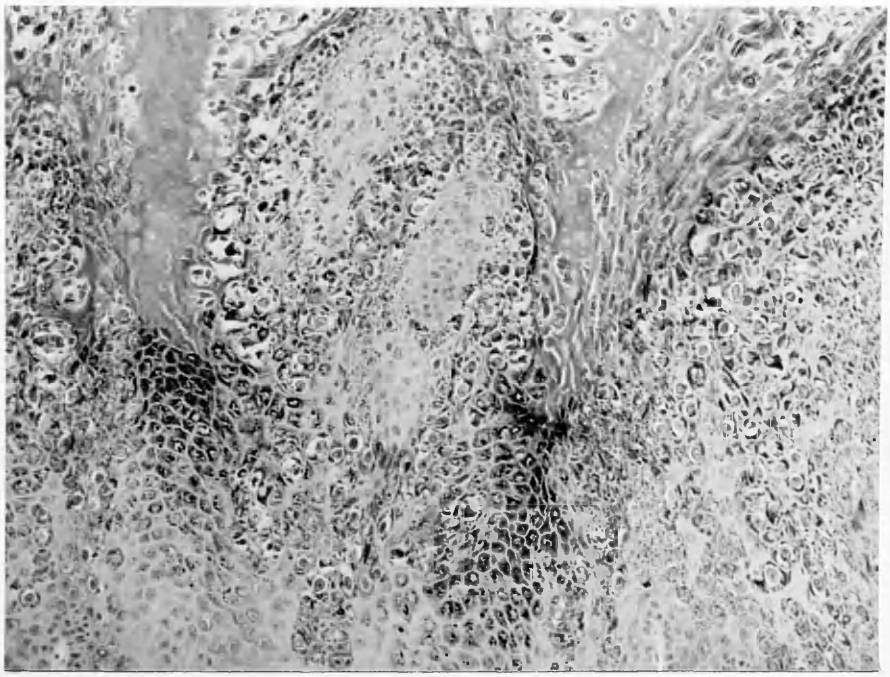


Fig. 17. Dyskeratotic papilloma; prominent keratohyalin granules in normal squamous cells with intervening masses of dyskeratotic cells.
Haematoxylin and eosin x 135.

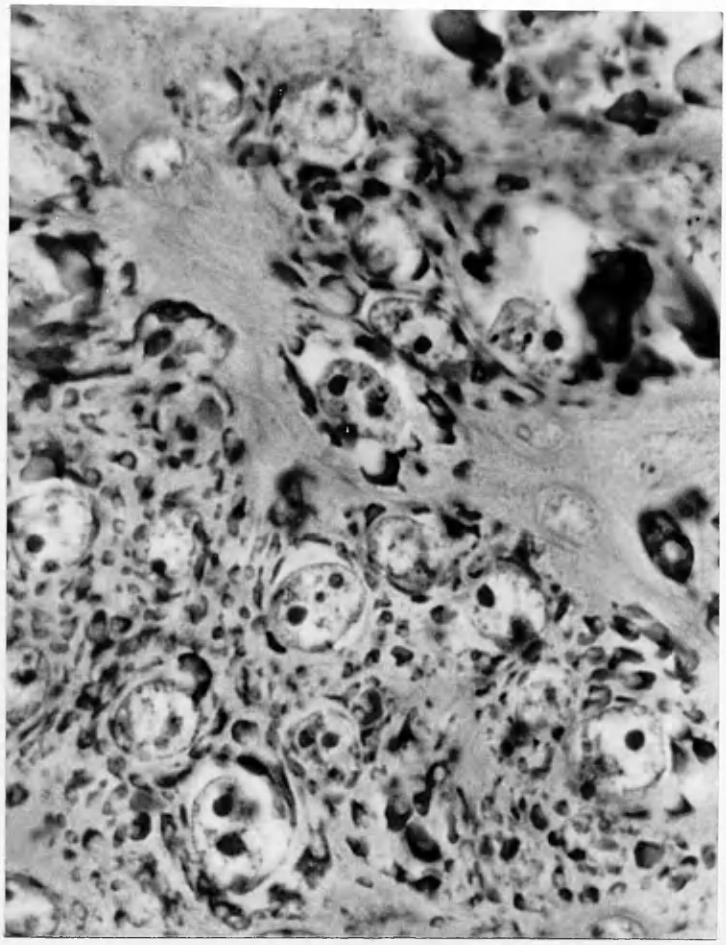


Fig. 18. Dyskeratotic papilloma; prominent intranuclear bodies, some nuclei also contain prominent nucleoli.
Haematoxylin and eosin x 950.

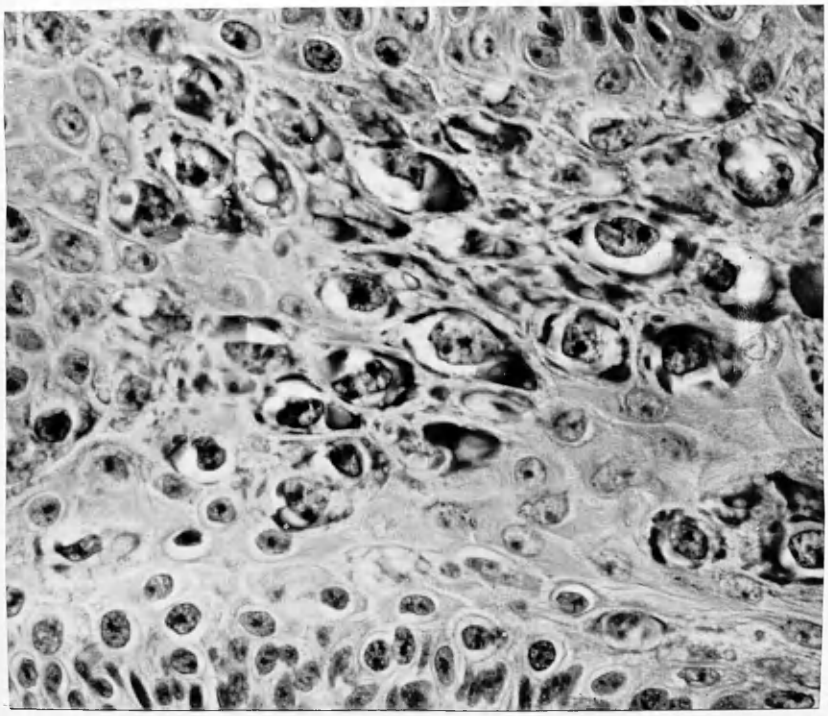


Fig. 19. Dyskeratotic papilloma; a group of dyskeratotic cells with adjacent normal squamous cells. Haematoxylin and eosin x 750.

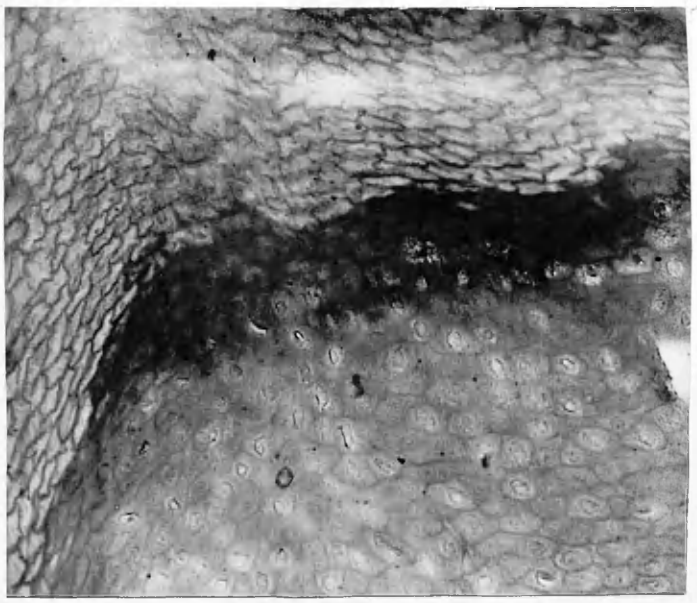


Fig. 20. Hyperkeratotic papilloma; intense staining for sulphydryl at the junction of the squamous and keratin layers.
Dihydroxy-dinapthyl-disulphide x 225.

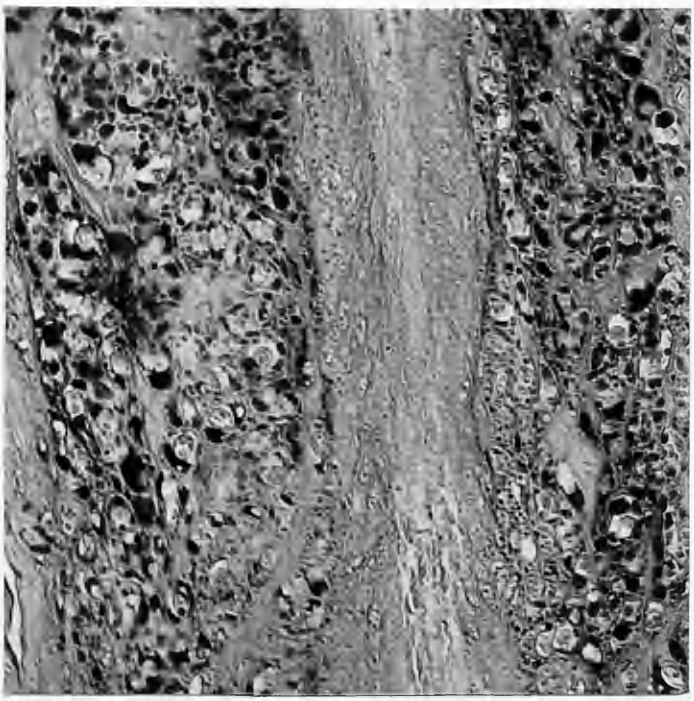


Fig. 21. Dyskeratotic papilloma; Sulphydryl containing dyskeratotic cells are separated by unreactive squamous cells.
Dihydroxy-dinapthyl-disulphide x 225.

Fig. 22. Dyskeratotic papilloma; the reaction for sulphydryl is present in the dyskeratotic cells and extends into the keratin layer. Dihydroxy-dinapthyl-disulphide x 90.

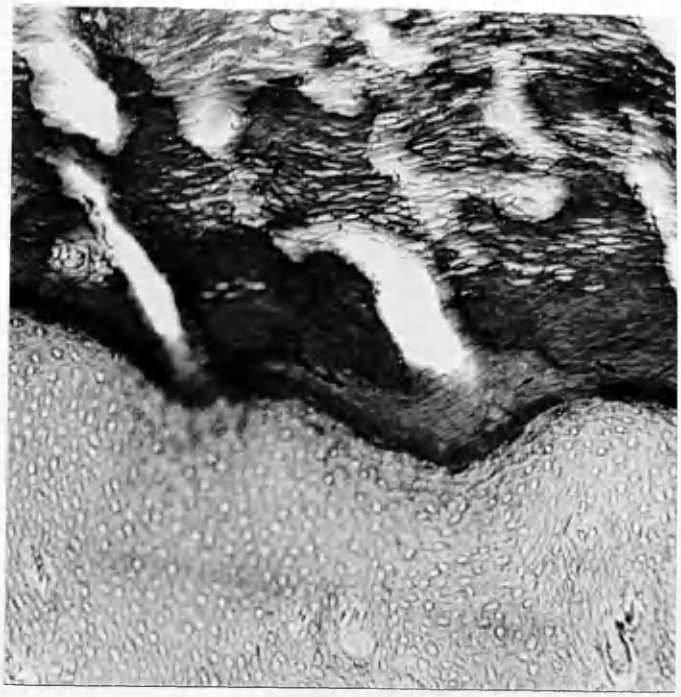


Fig. 23. Hyperkeratotic papilloma; staining for disulphide is confined to the keratin layer.
Dihydroxy-dinapthyl-disulphide x 135.

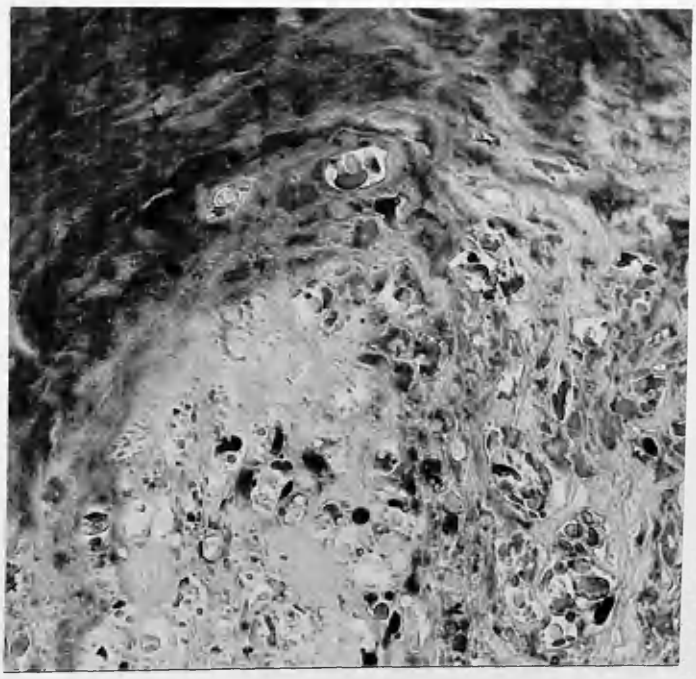


Fig. 24. Dyskeratotic papilloma; staining for disulphide is confined to the dyskeratotic cells and the keratin layer.
Dihydroxy-dinapthyl-disulphide x 225.

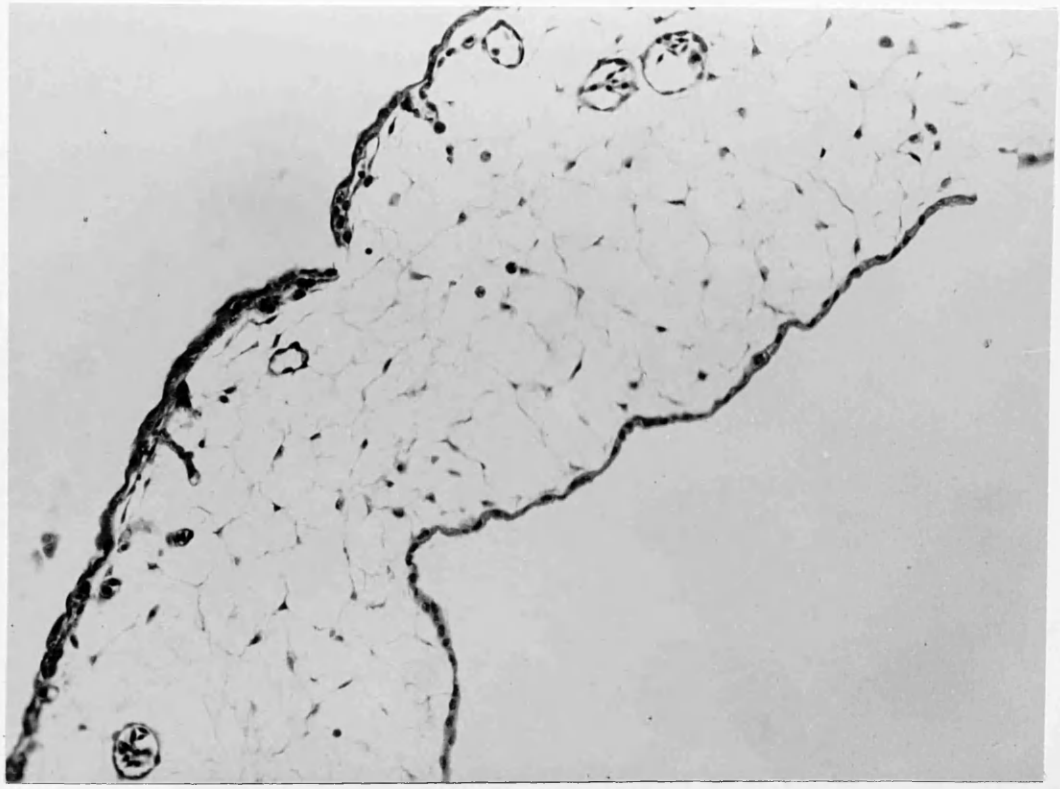


Fig. 25. Normal chick chorio-allantoic membrane. The superficial ectoderm consists of flattened epithelial cells. Haematoxylin and eosin x 275.

Fig. 26. Membrane inoculated with extracts of normal skin; the ectoderm is now squamous in type. Haematoxylin and eosin x 275.

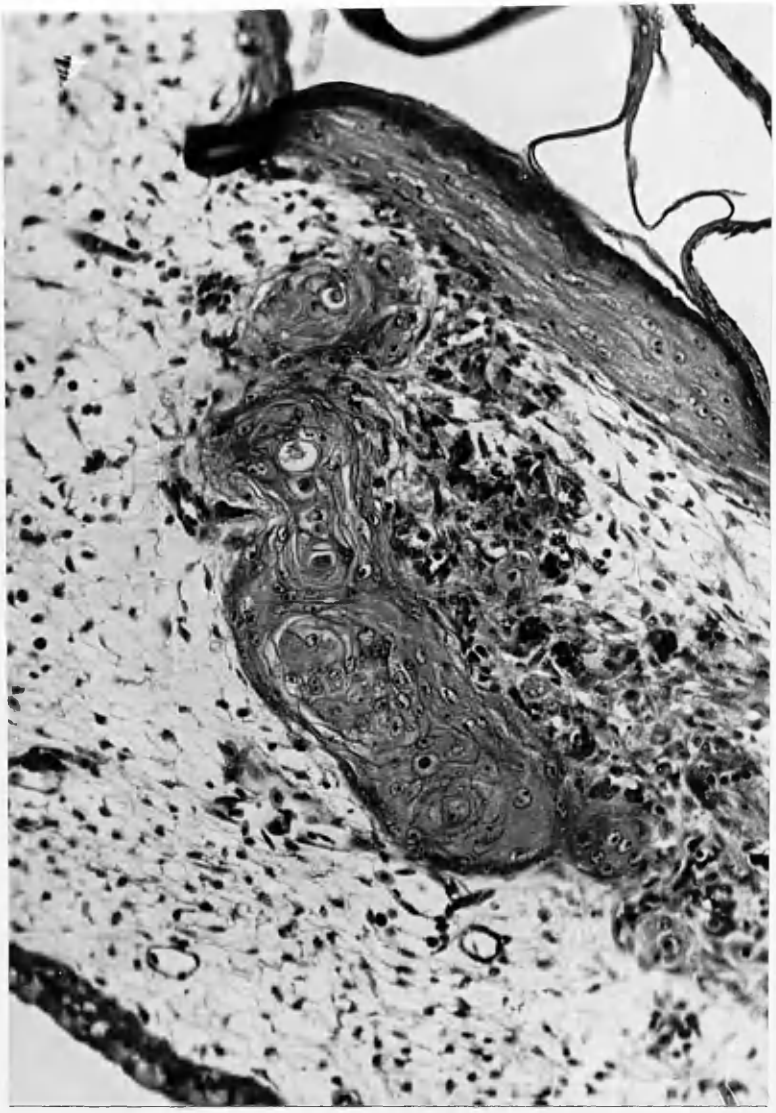


Fig. 27. Membrane inoculated with wart material. There is invasion of the mesoderm by "cell nests". Haematoxylin and eosin x 275.

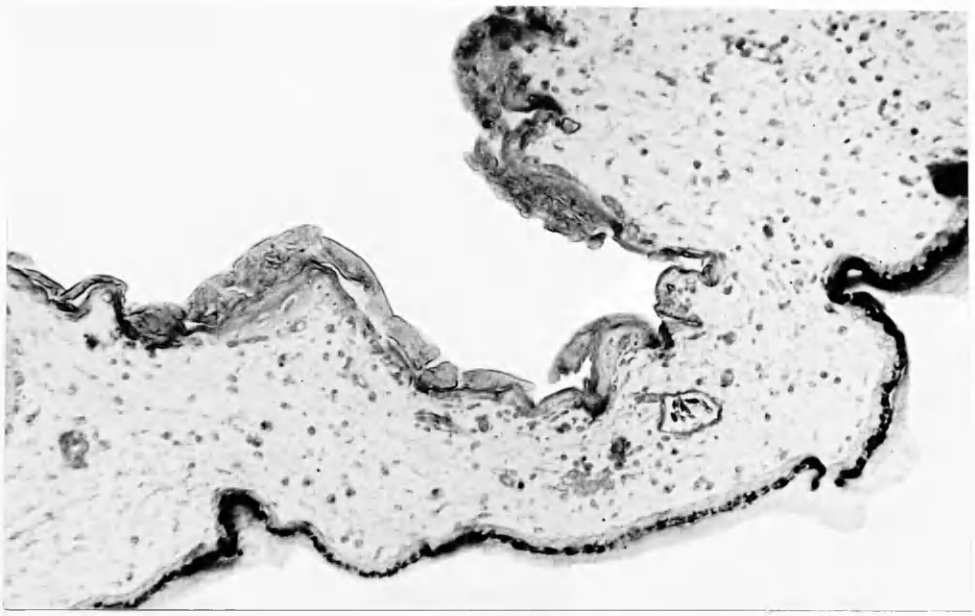


Fig. 28. Chorio-allantoic membrane; glycogen is confined to the endoderm.
Periodic acid-Schiff x 230.

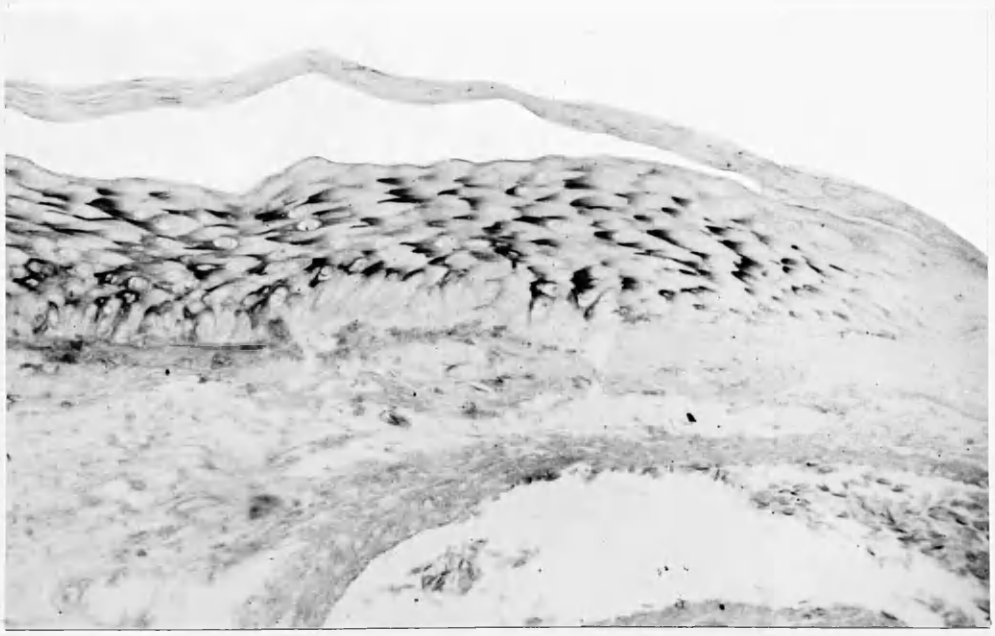


Fig. 29. Hyperplastic membrane; glycogen in the ectoderm.
Periodic acid-Schiff x 230.

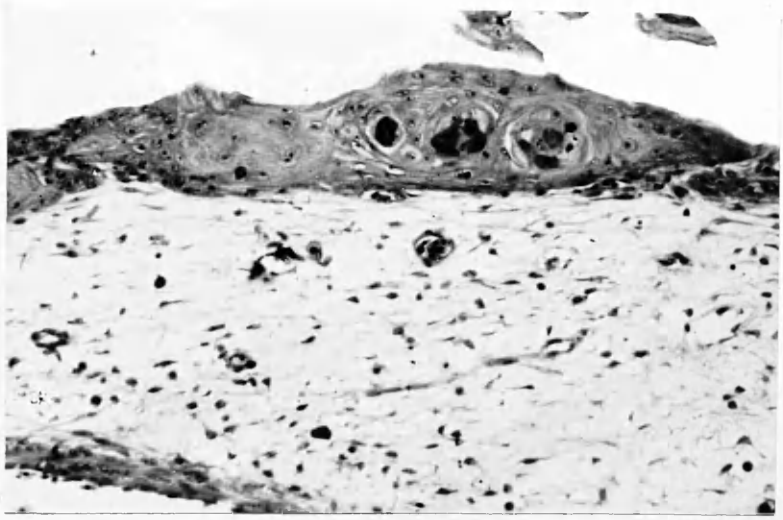


Fig. 30. Hyperplastic membrane; sulphydryl groups in the ectoderm.
Dihydroxy-dinapthyl-disulphide x 230.

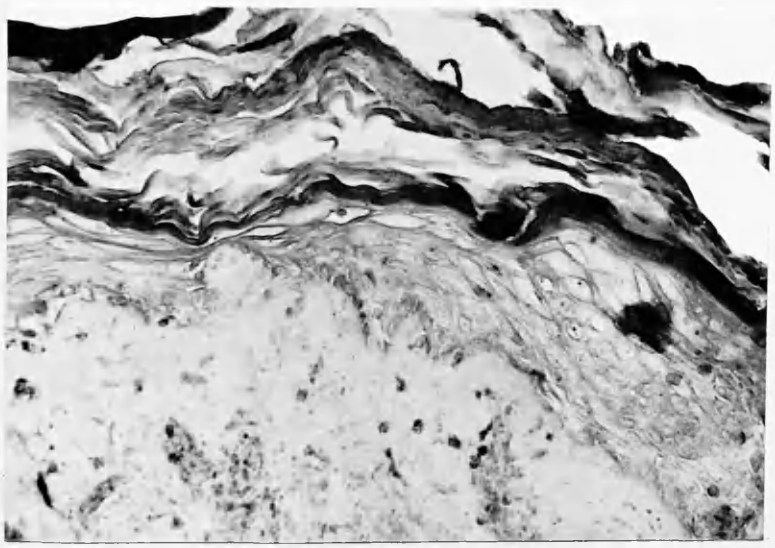


Fig. 31. Hyperplastic membrane; sulphydryl groups in the superficial squamous and keratin layers.
Dihydroxy-dinapthyl-disulphide x 230.

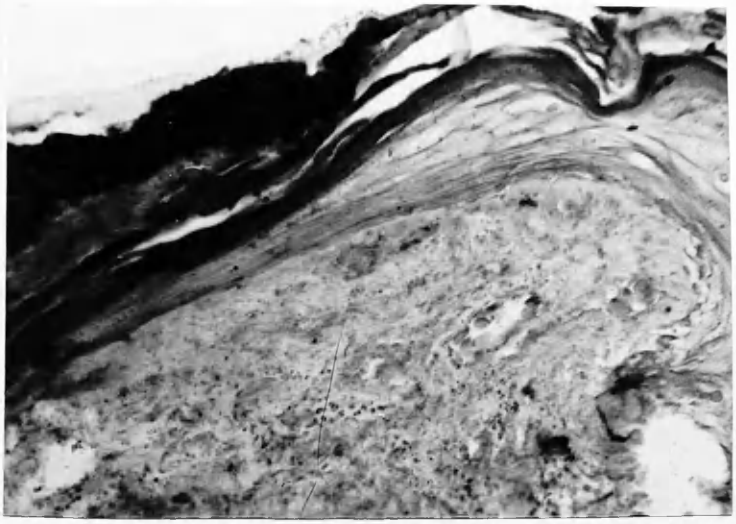


Fig. 32. Hyperplastic membrane; disulphide is confined to the keratin layer.
Dihydroxy-dinapthyl-disulphide x 230.

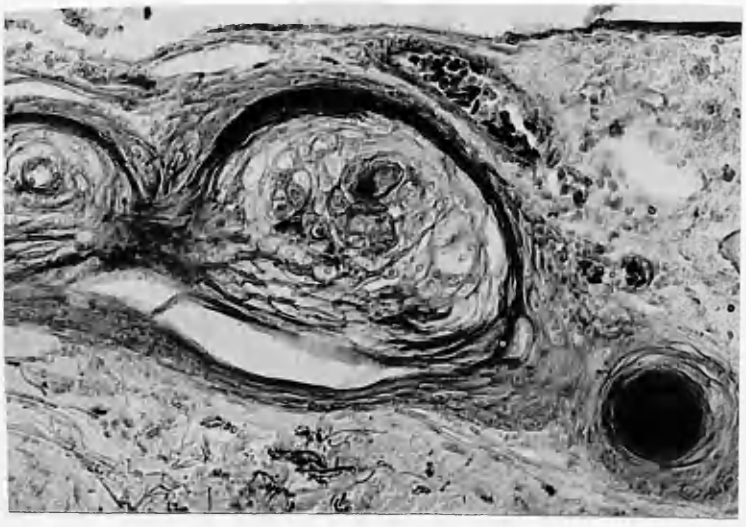


Fig. 33. Hyperplastic membrane; intense staining for disulphide in "cell nests".
Dihydroxy-dinapthyl-disulphide x 230.

Fig. 34. Human skin grafted on the chorio-allantoic membrane.
x 4.

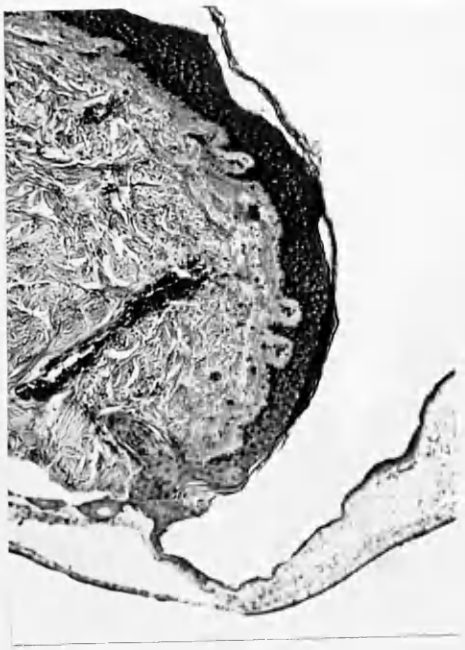


Fig. 35. Human skin grafted on the membrane.
Haematoxylin and eosin x 30.

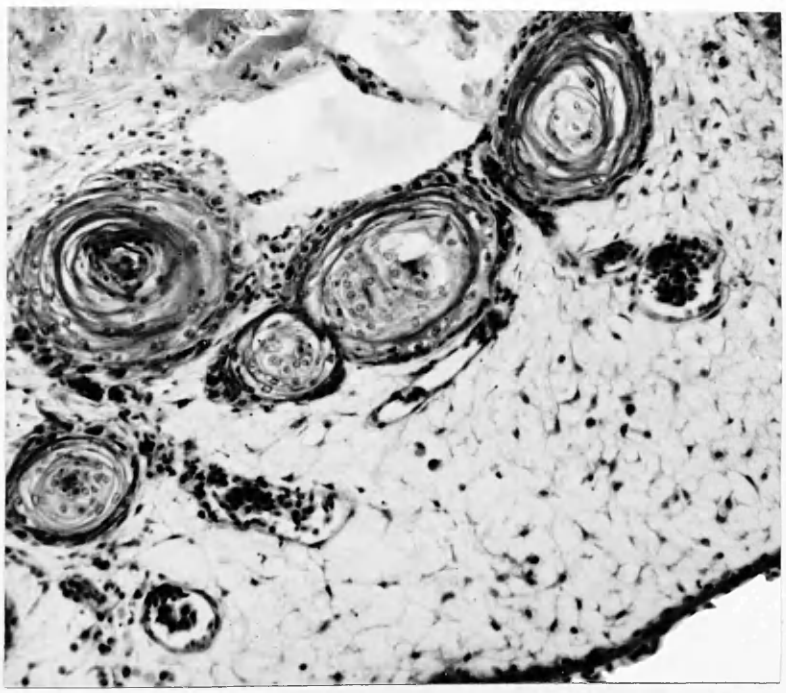


Fig. 36. Human skin grafted on the membrane; "cell nests" are formed at the base of the graft. Haematoxylin and eosin x 230.

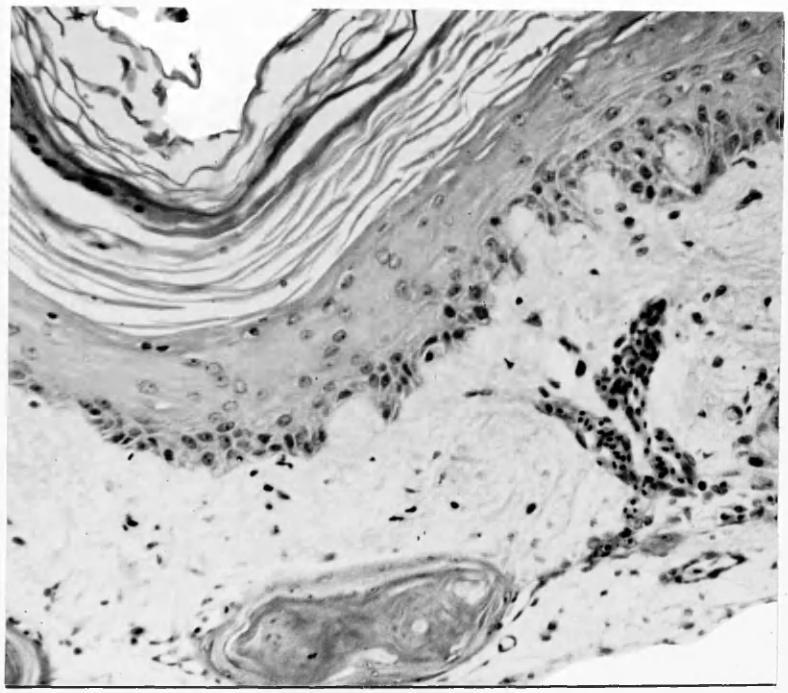


Fig. 37. Keratinising human skin grafted on the membrane. Nucleated chick red cells are present in the dermis of the graft.
Haematoxylin and eosin x 230.

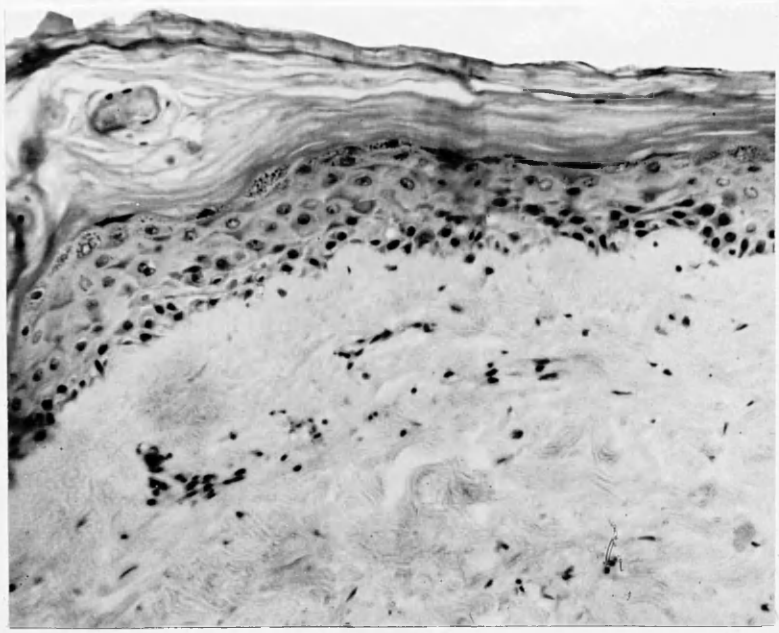


Fig. 38. Human skin grafted on the membrane; abundant keratin is present but keratohyalin granules are scanty.
Haematoxylin and eosin x 230.

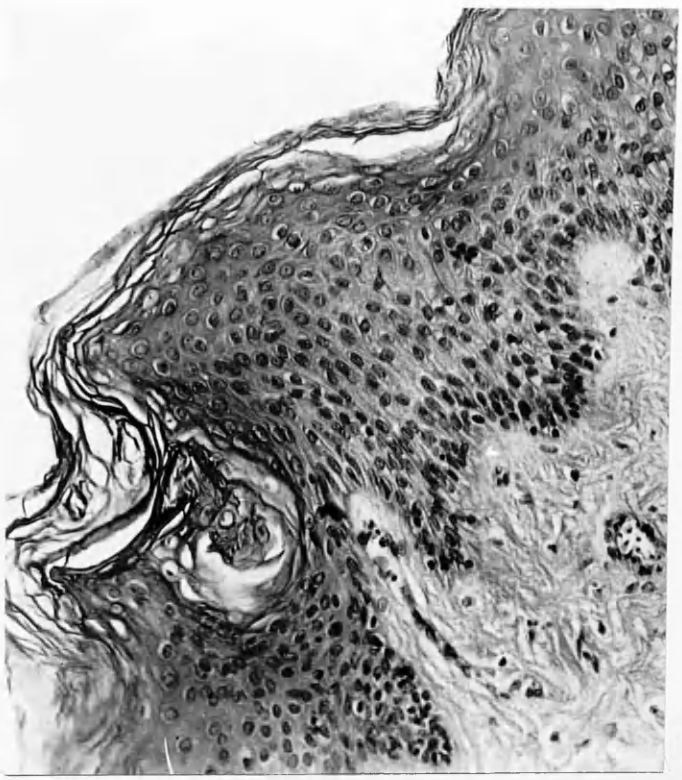


Fig. 39. Human skin graft inoculated with wart material. There is hyperplasia of the squamous cell layer and hyperkeratosis. Haematoxylin and eosin x 230.

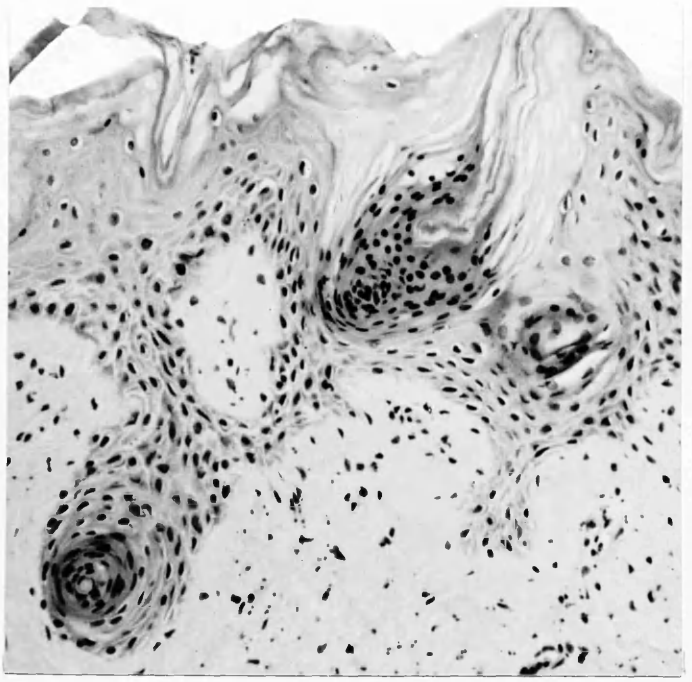


Fig. 40. Human skin graft inoculated with wart material; hyperkeratosis and parakeratosis. Haematoxylin and eosin x 230.

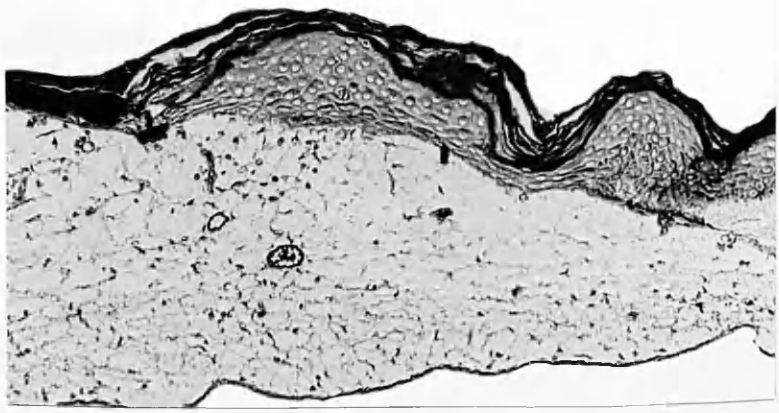


Fig. 41. Human skin graft inoculated with wart material. Sulphydryl staining is most intense in the keratin layers.
Dihydroxy-dinapthyl-disulphide x 230.

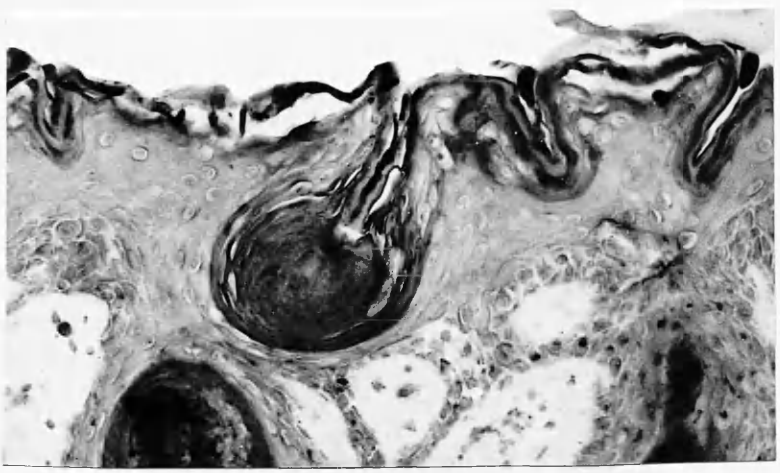


Fig. 42. Human skin graft inoculated with wart material. Intense sulphydryl staining in the central keratin.
Dihydroxy-dinapthyl-disulphide x 230.

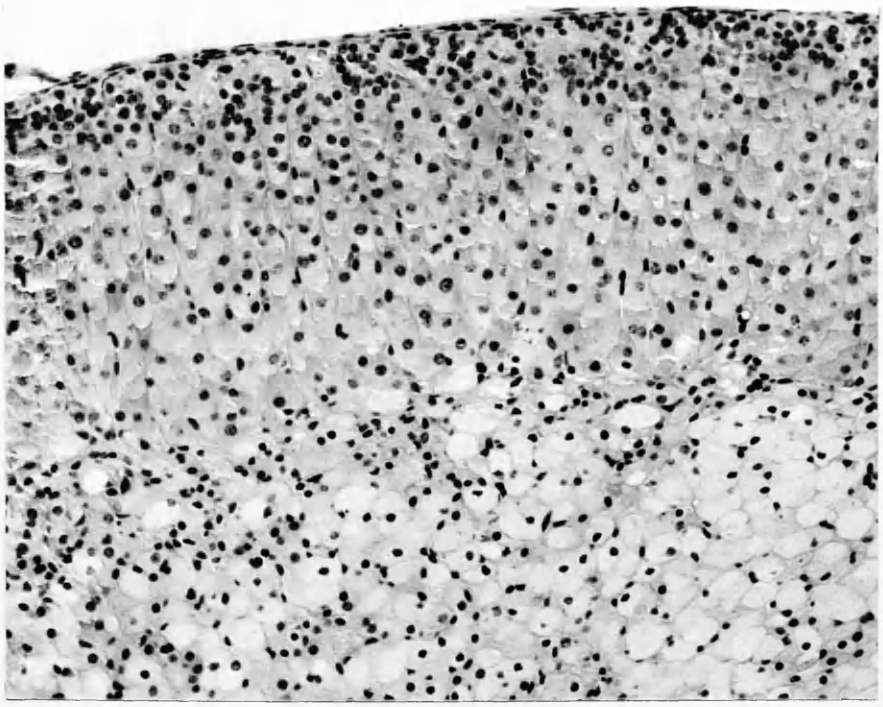


Fig. 43. Adrenal of a mouse bearing a corticotrophin secreting tumour. The Z. fasciculata and reticularis are enlarged.
Haematoxylin and eosin x 225.

Fig. 44. LAF1 mice. The larger animal bears a corticotrophin secreting tumour.

Fig. 45. LAF 1 mouse; corticotrophin secreting tumour growing in thigh muscle.

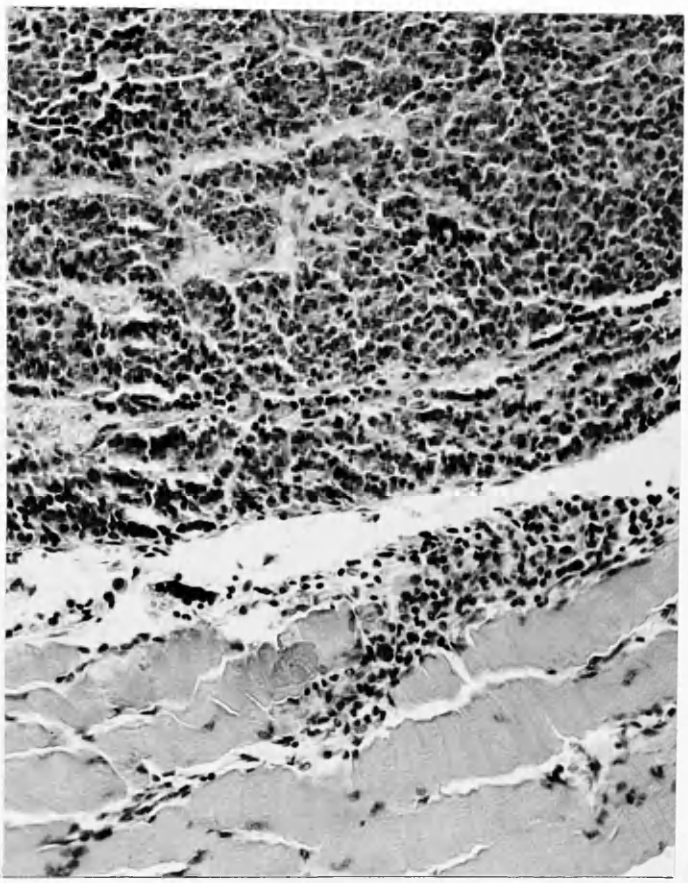


Fig. 46. LAF 1 mouse; corticotrophin secreting tumour growing in thigh muscle. Haematoxylin and eosin x 225.

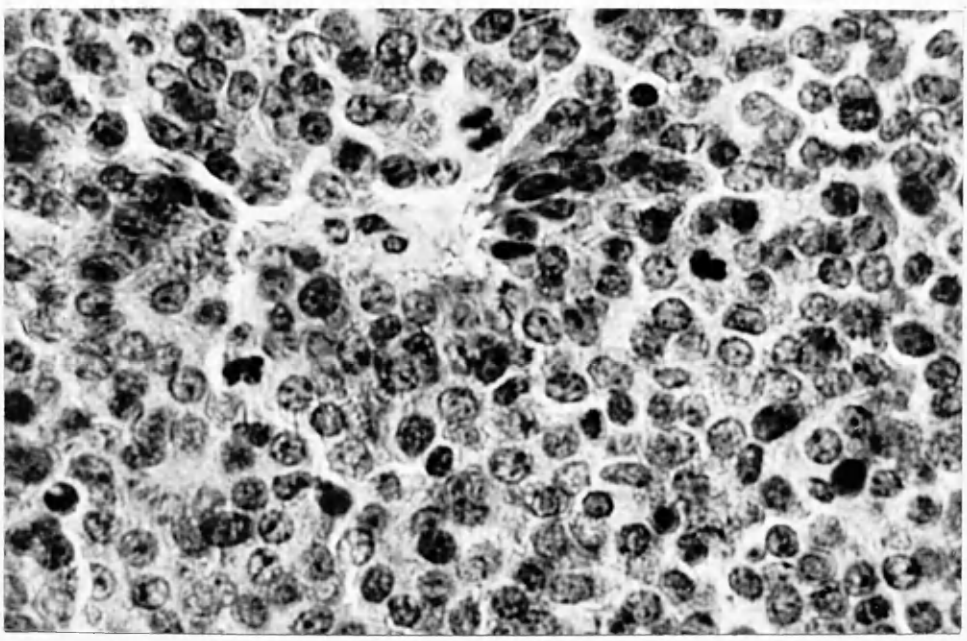


Fig. 47. Corticotrophin secreting tumour. The cells are uniform in type and show moderate mitotic activity. Haematoxylin and eosin x 950.

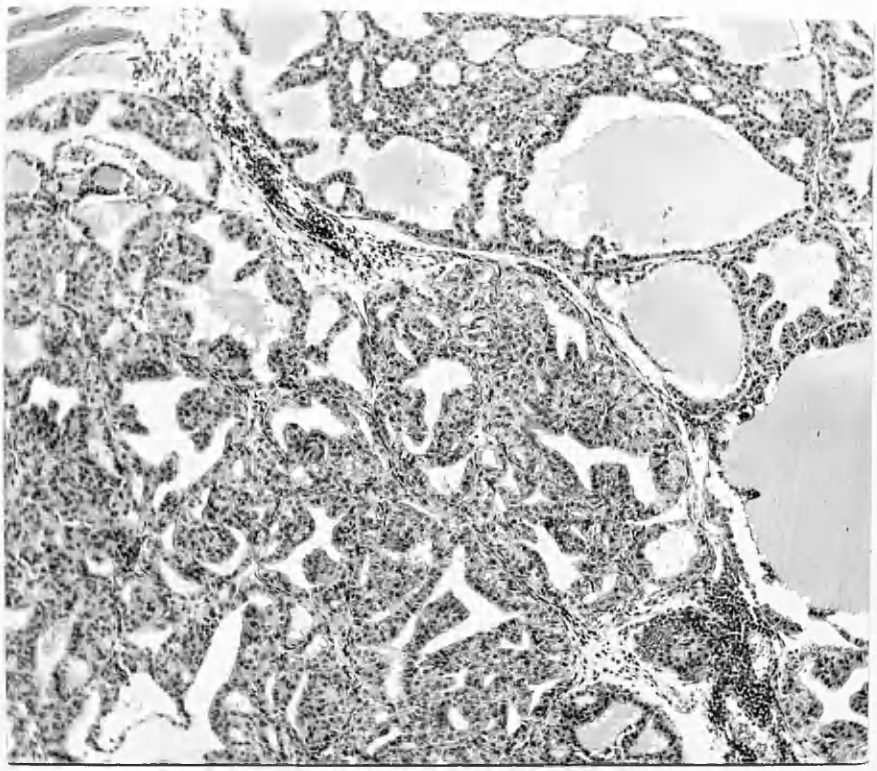


Fig. 48. Thyroid of LAF1 mouse which bears a thyrotrophin secreting tumour; widespread epithelial hyperplasia.
Haematoxylin and eosin x 225.

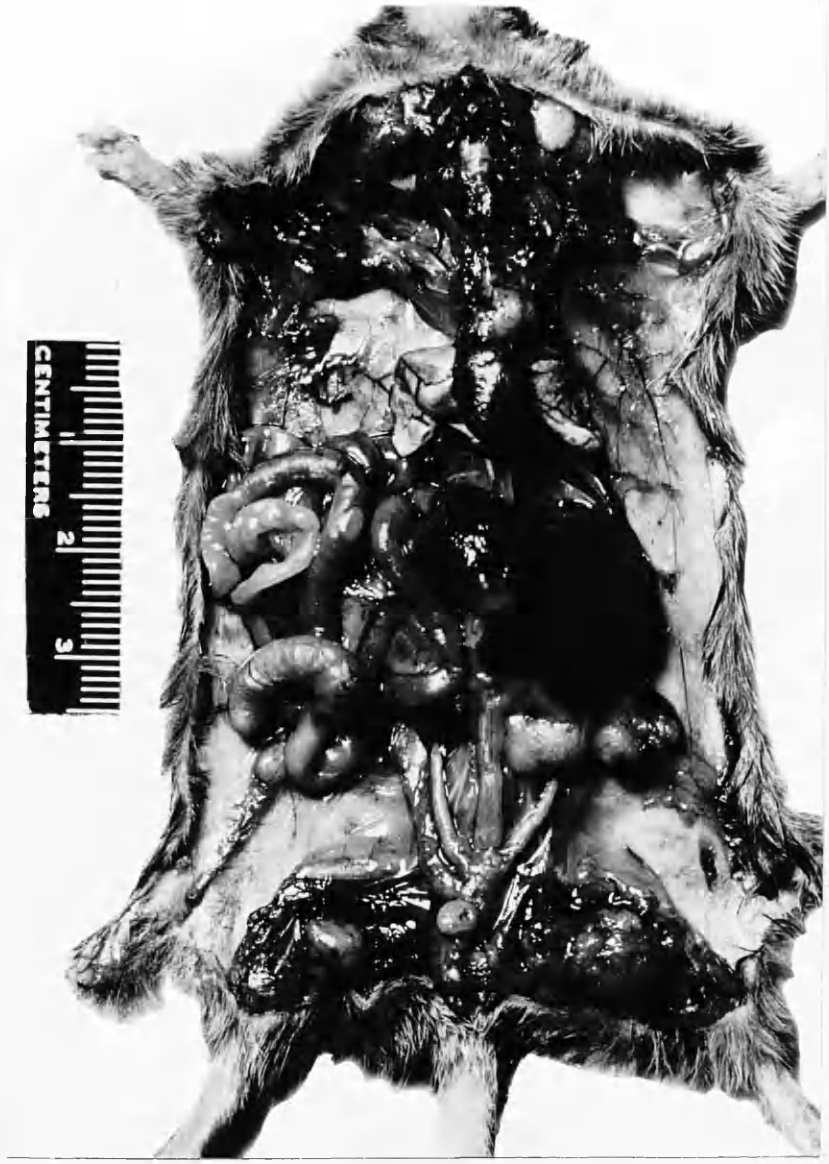


Fig. 49. LAF 1 mouse with thyrotrophin secreting tumours in both thighs; marked enlargement of ovaries and uterine horns.

Fig. 50. Portion of a grossly dilated uterine horn from a mouse with a thyrotrophin secreting tumour. Haematoxylin and eosin x 135.

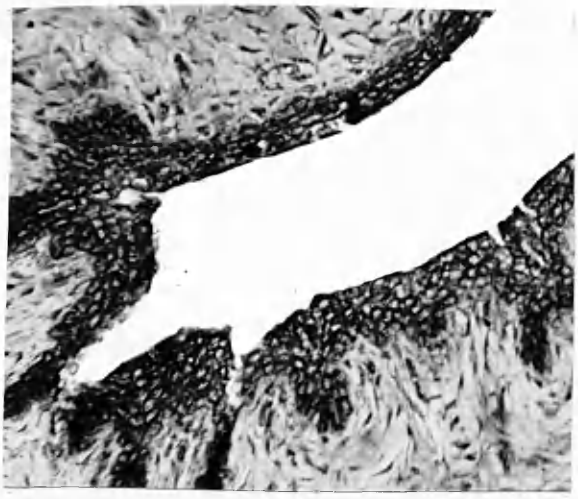


Fig. 51. Sulphydryl groups in non-keratinised di-oestrus vagina.
Dihydroxy-dinapthyl-disulphide x 225.

Fig. 52. Sulphydryl groups in upper squamous and keratin layers in oestrus vagina.
Dihydroxy-dinapthyl-disulphide x 225.

Fig. 53. Disulphide groups in keratin layer in oestrus vagina.
Dihydroxy-dinapthyl-disulphide x 225.

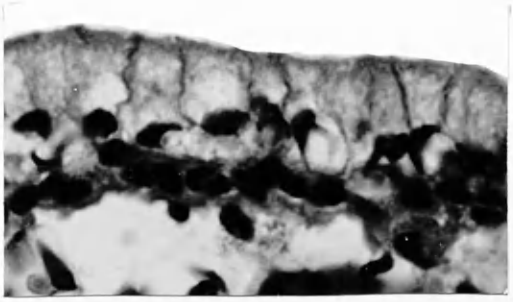


Fig. 54. Vaginal epithelium of a mouse with a corticotrophin secreting tumour; a superficial layer of tall cuboidal cells.
Haematoxylin and eosin x 950.

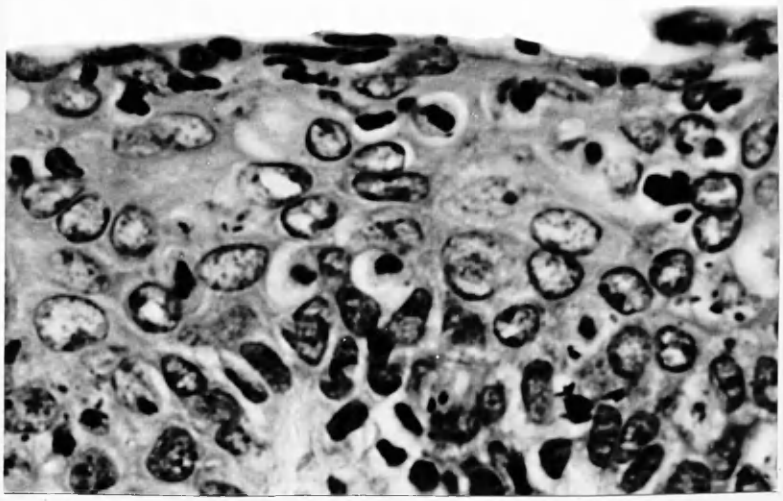


Fig. 55. Vaginal epithelium of a mouse with a corticotrophin secreting tumour; stratified squamous cells but no keratin.
Haematoxylin and eosin x 950.

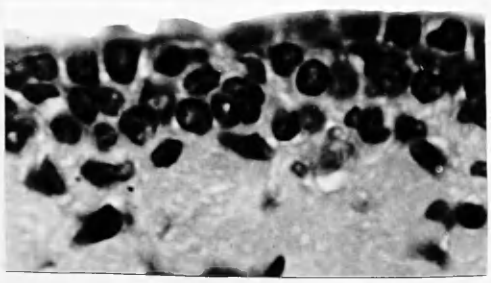


Fig. 56. Vaginal epithelium from a gonadectomised and adrenalectomised mouse; a double layer of cuboidal cells.
Haematoxylin and eosin x 950.

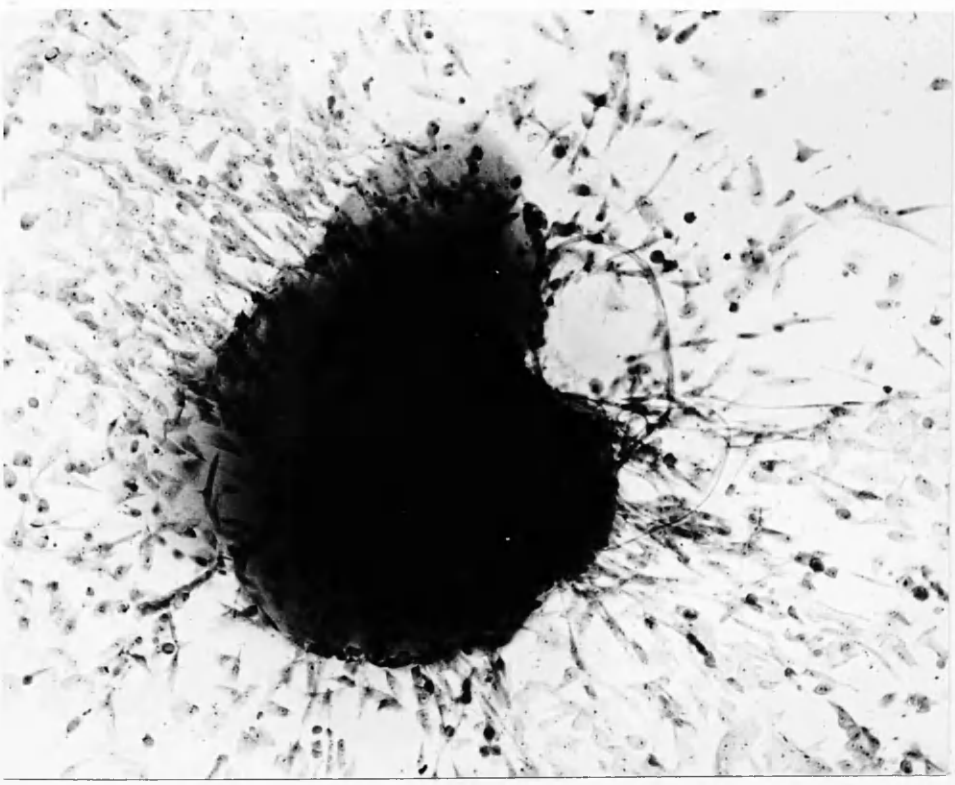


Fig. 57. Explant of human adult skin; the cells are mainly fibroblasts.
Giemsa x 90.

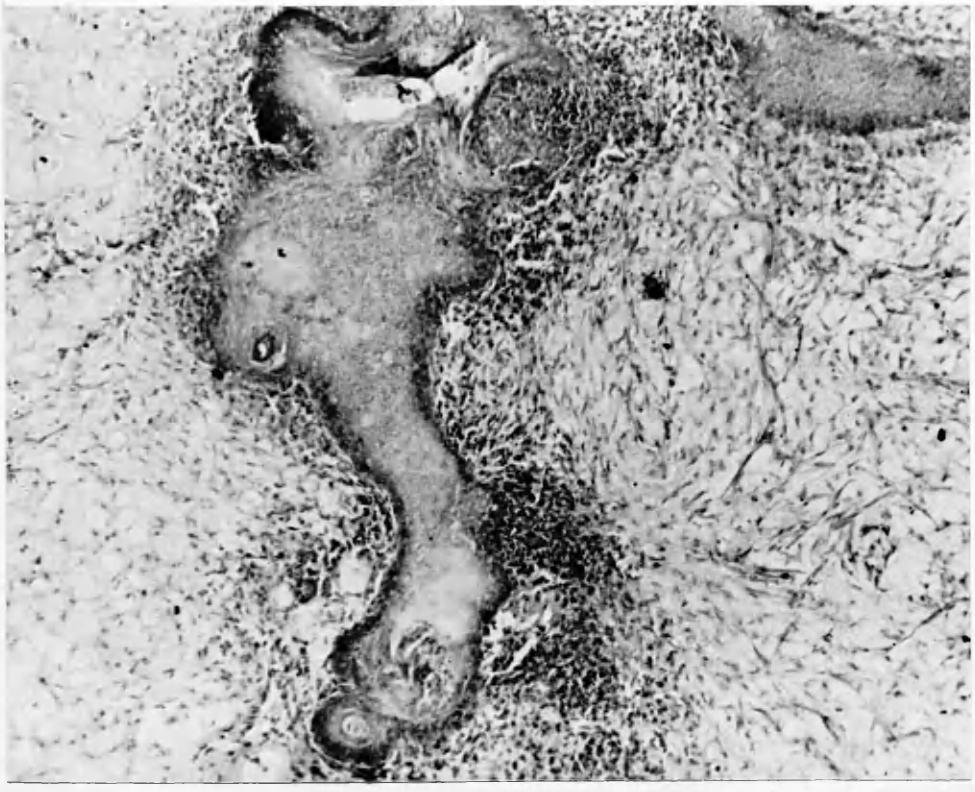


Fig. 58. Explant of human adult skin; mixed growth of fibroblasts and epithelial cells.
Giemsa x 90.

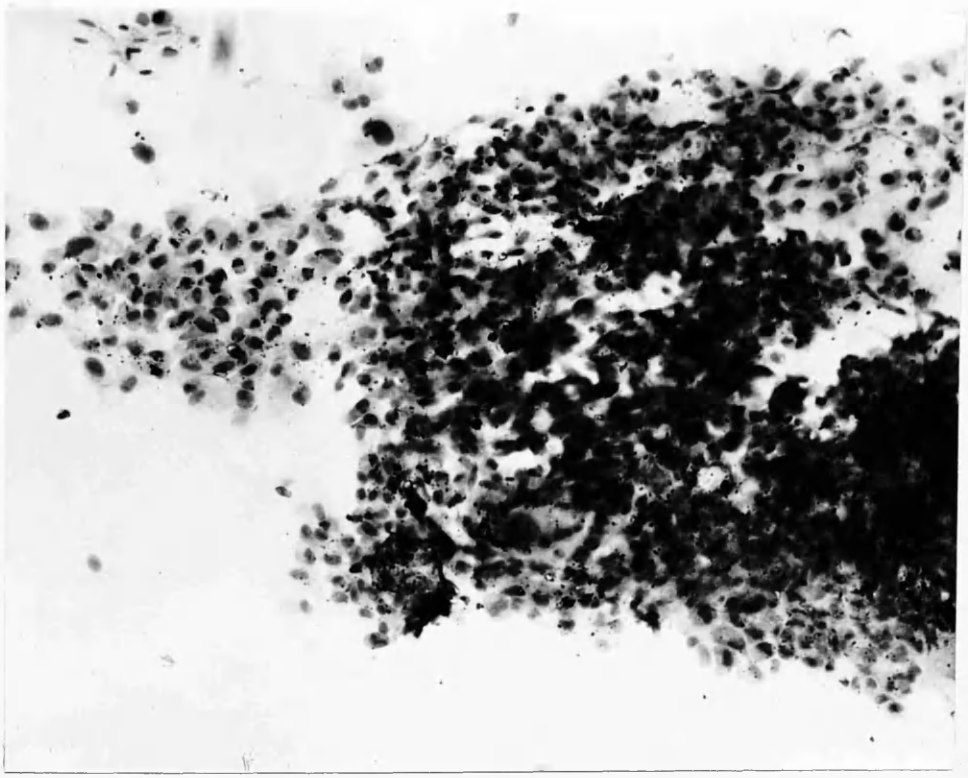


Fig. 59. Outgrowth from human skin; the cells are epithelial in type.
Giemsa x 140.

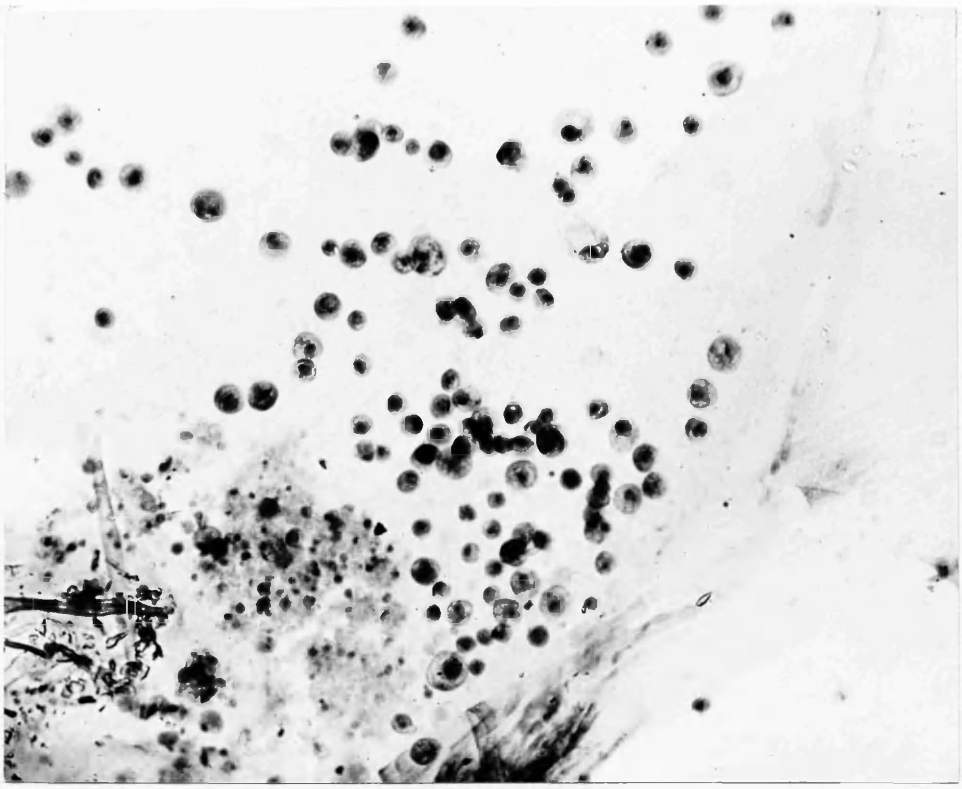


Fig. 60. Degenerating epithelial cells; there is no evidence of keratin formation.
Giemsa x 140.

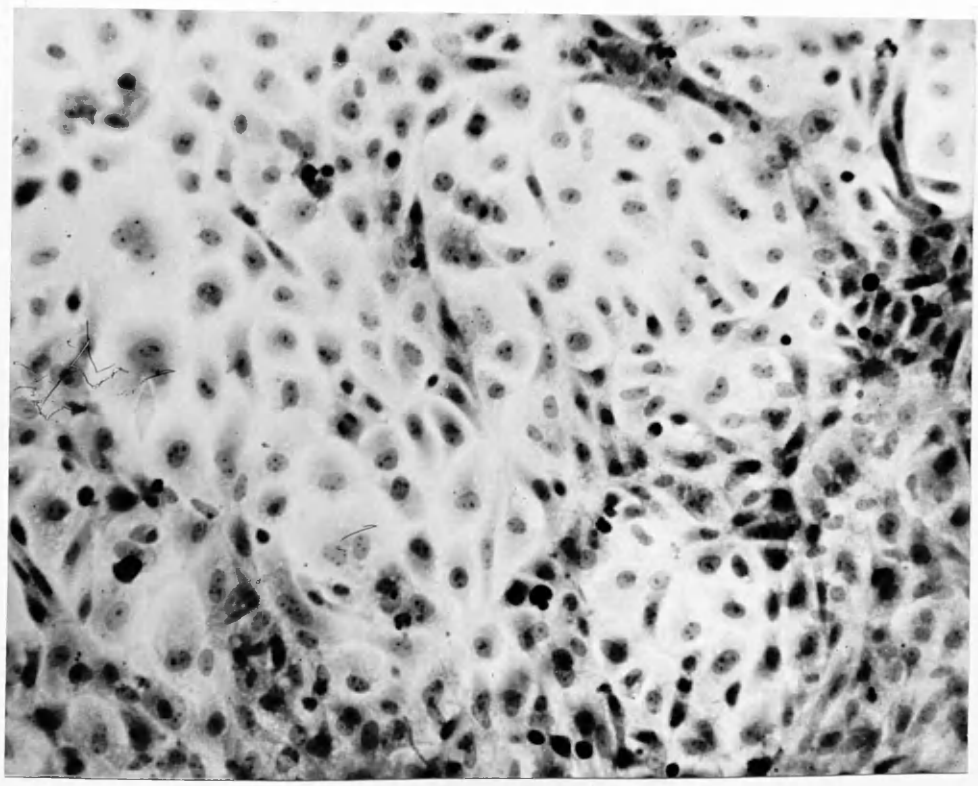


Fig. 61. Subculture of an outgrowth from human adult skin; the cells are still epithelial in type.
Giemsa x 230.

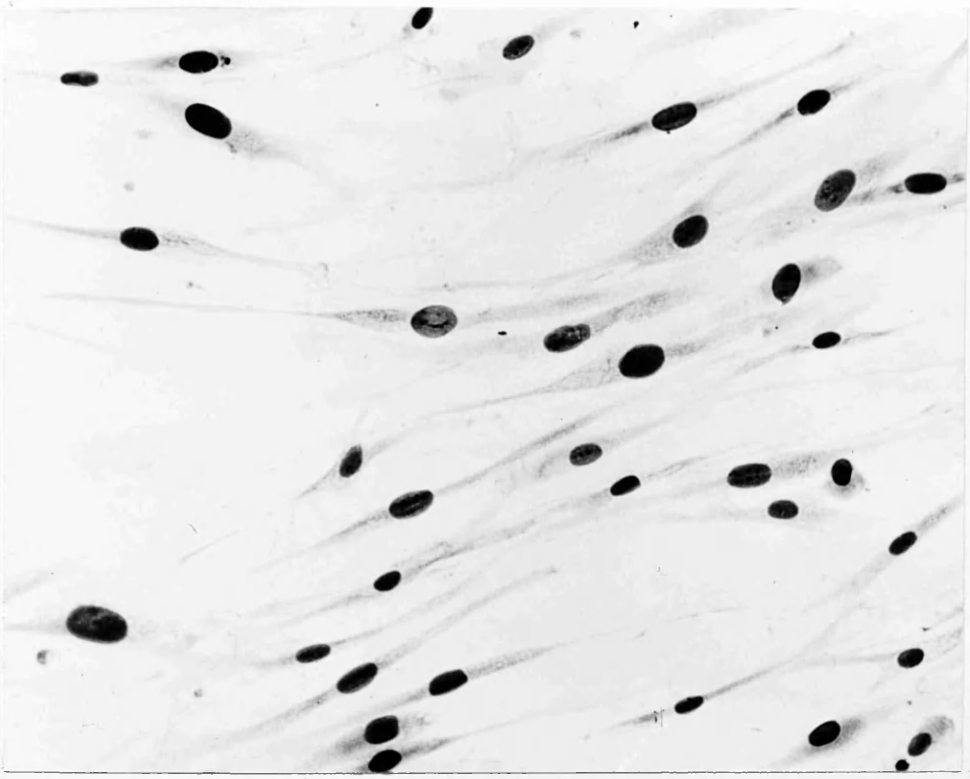


Fig. 62. Subculture of the epithelial cells shown in fig. 61; the cells are now indistinguishable from fibroblasts. Giemsa x 230.

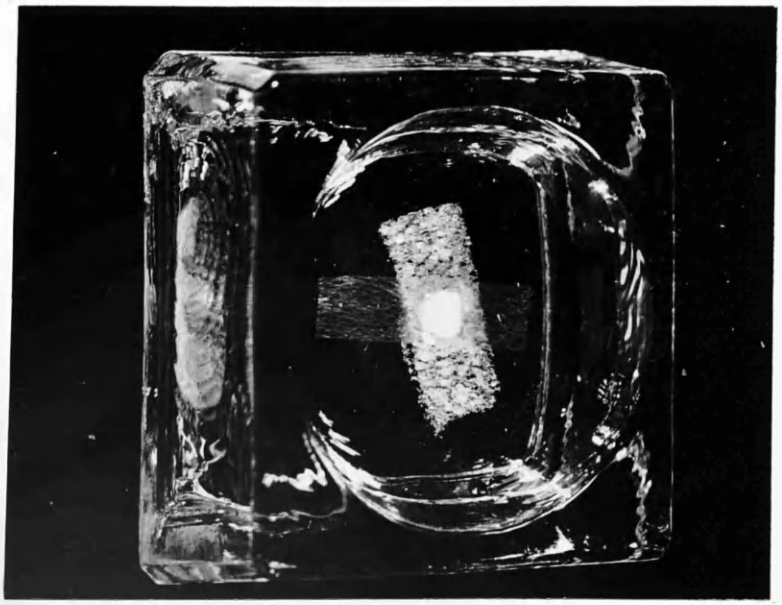


Fig. 63. Human adult skin in culture dish. The tissue rests on lens paper and is supported by a block of sponge.

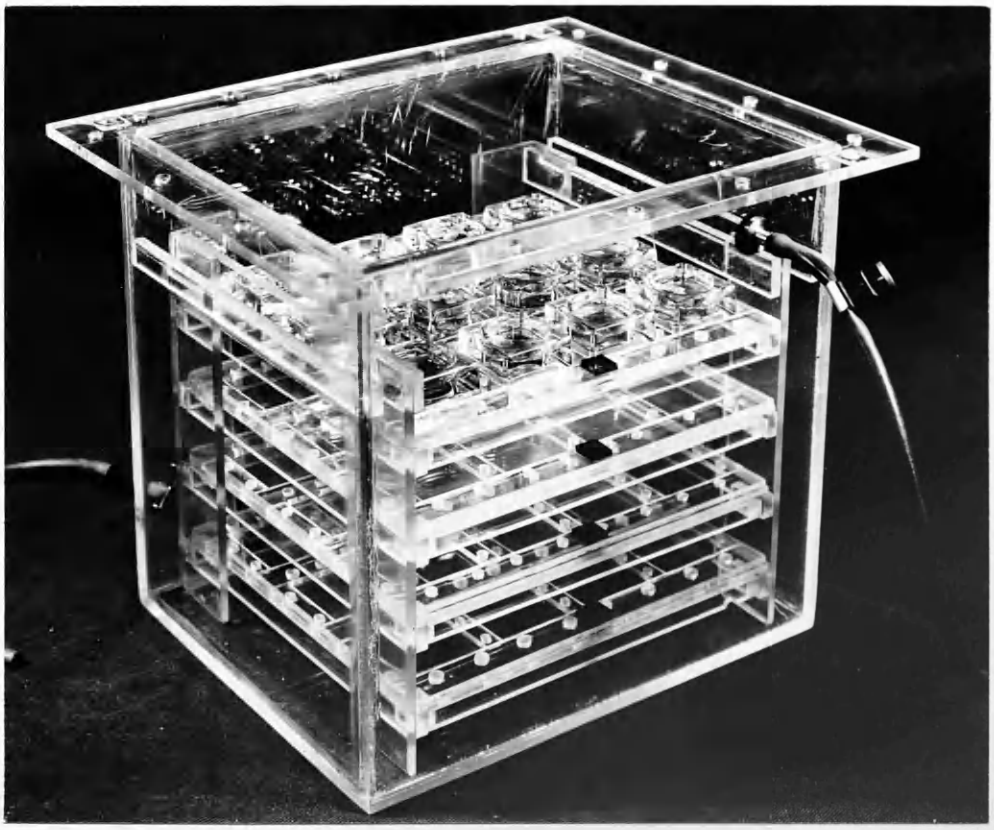


Fig. 64. Perspex culture chamber showing 12 culture dishes in use.

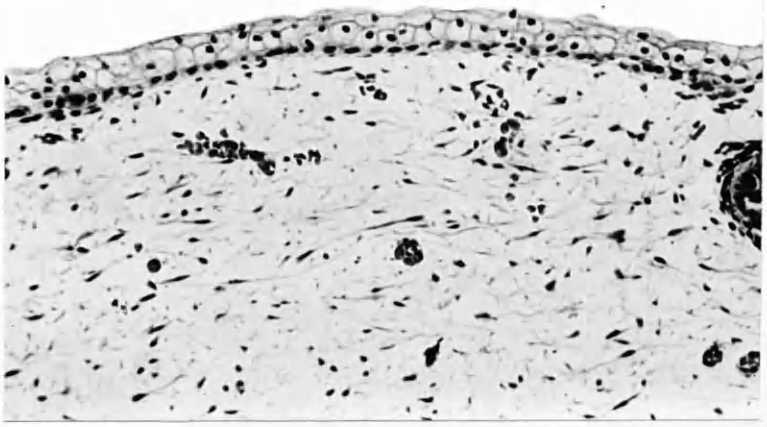


Fig. 65. Human foetal skin before culture; the S. intermedium consists of large cuboidal cells. Haematoxylin and eosin x 225.

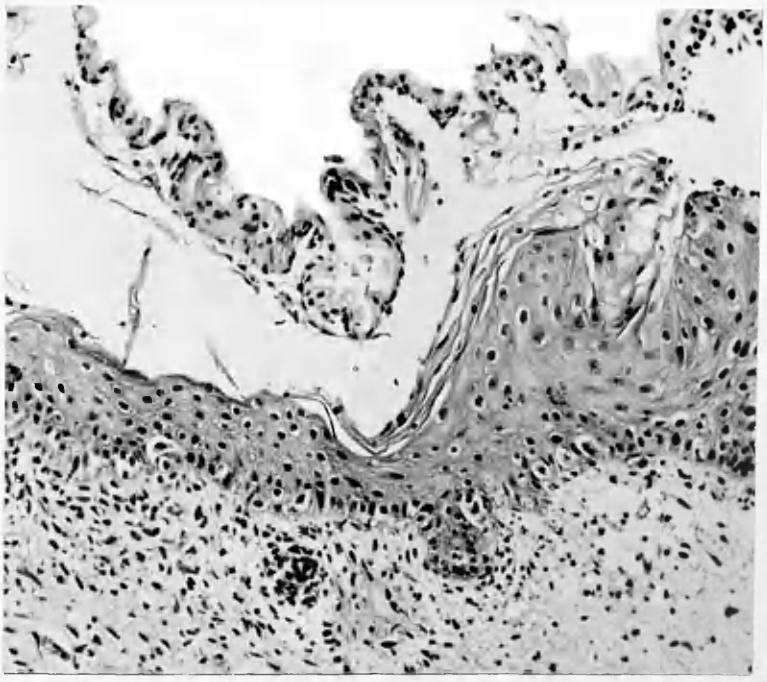


Fig. 66. Human foetal skin after 8 days in culture; the epidermis is now composed of squamous cells and covered by a thin layer of keratin in which there are parakeratotic cells.
Haematoxylin and eosin x 225.

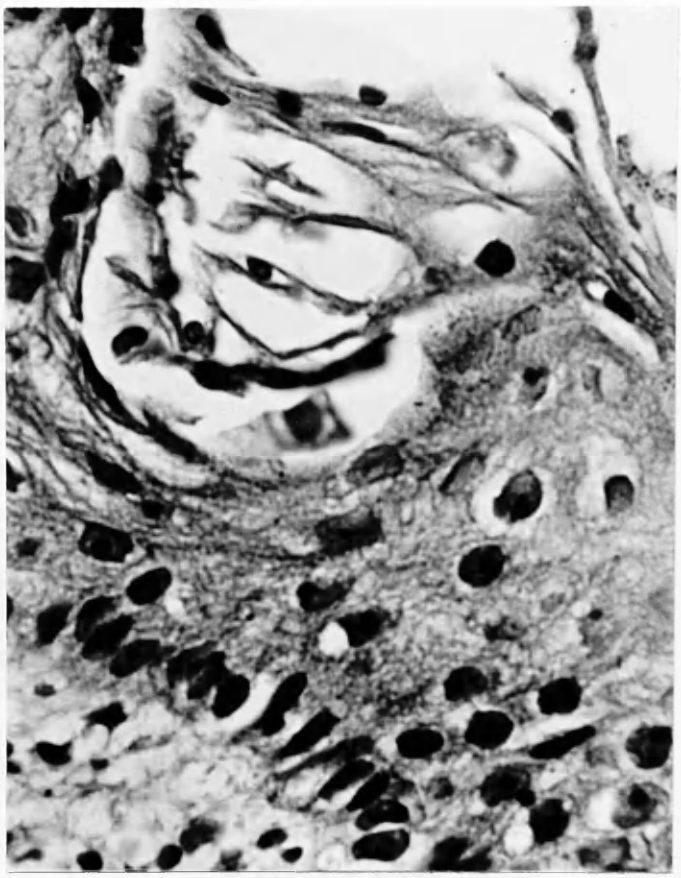


Fig. 67. Human foetal skin after 8 days in culture; an area of parakeratosis. Haematoxylin and eosin x 950.

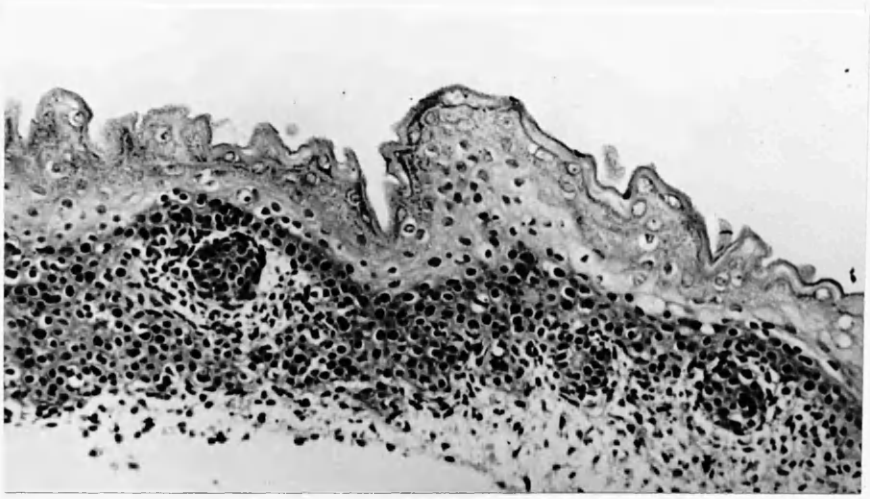


Fig. 68. Human foetal skin cultured in the presence of vitamin A for 8 days. Basal cell proliferation and prominent keratohyalin granules.
Haematoxylin and eosin x 225.

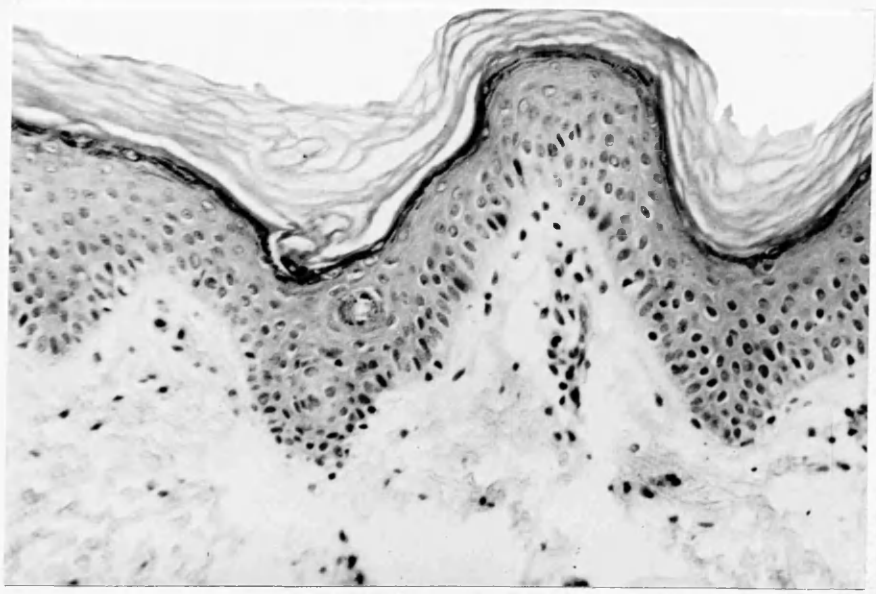


Fig. 69. Adult human skin before culture; there is a loosely attached keratin layer.
Haematoxylin and eosin x 230.

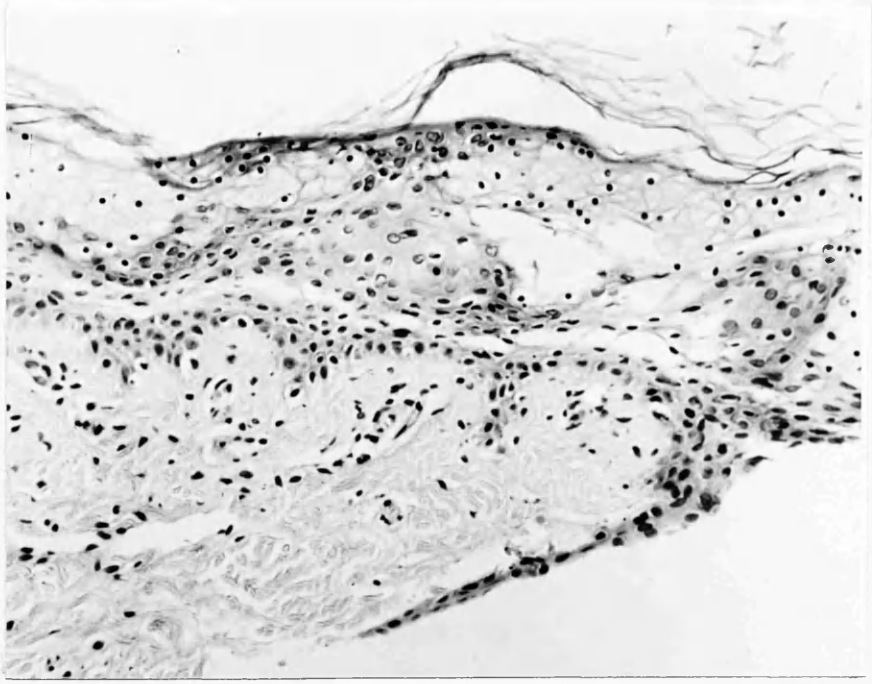


Fig. 70. Adult human skin after 2 days in culture; there is some separation of the epidermis which is composed of actively proliferating cells. Haematoxylin and eosin x 225.

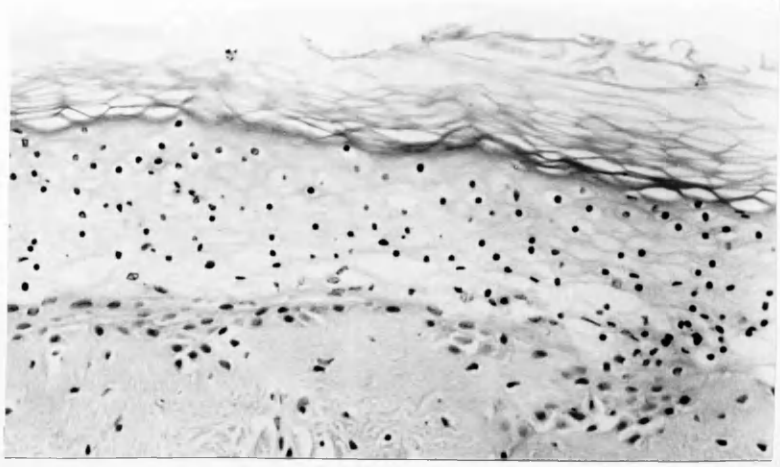


Fig. 71. Adult human skin after 4 days in culture; the epidermis is composed of a thin layer of spindle shaped cells and parakeratotic cells.
Haematoxylin and eosin x 225.

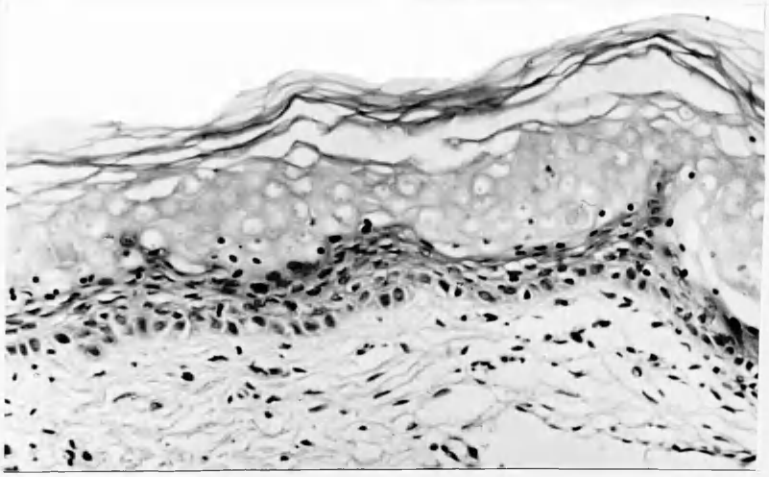


Fig. 72. Adult human skin after 6 days in culture; there is now a broad zone of acellular keratin.
Haematoxylin and eosin x 225.

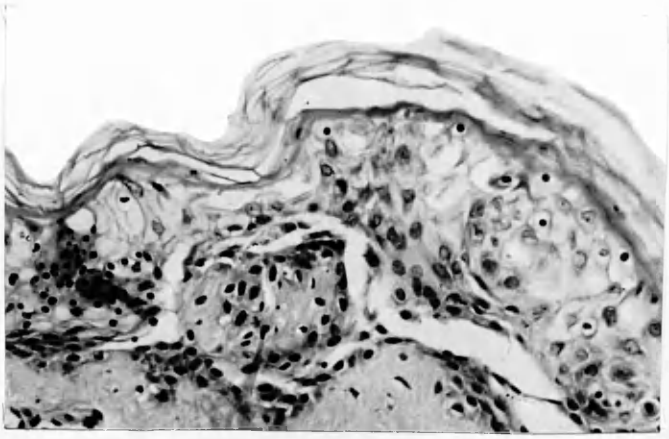


Fig. 73. Adult human skin cultured with oestradiol; after 2 days the appearances are similar to control cultures after 4 days.
Haematoxylin and eosin x 225.

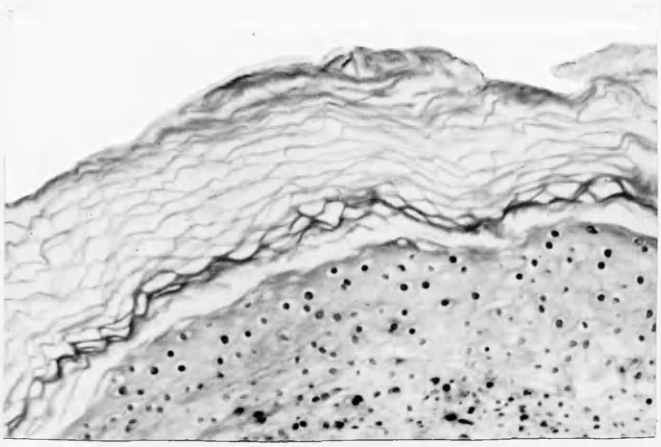


Fig. 74. Adult human skin cultured with oestradiol; at 6 days the pre-existing squamous cell layer is completely keratinised.
Haematoxylin and eosin x 225.

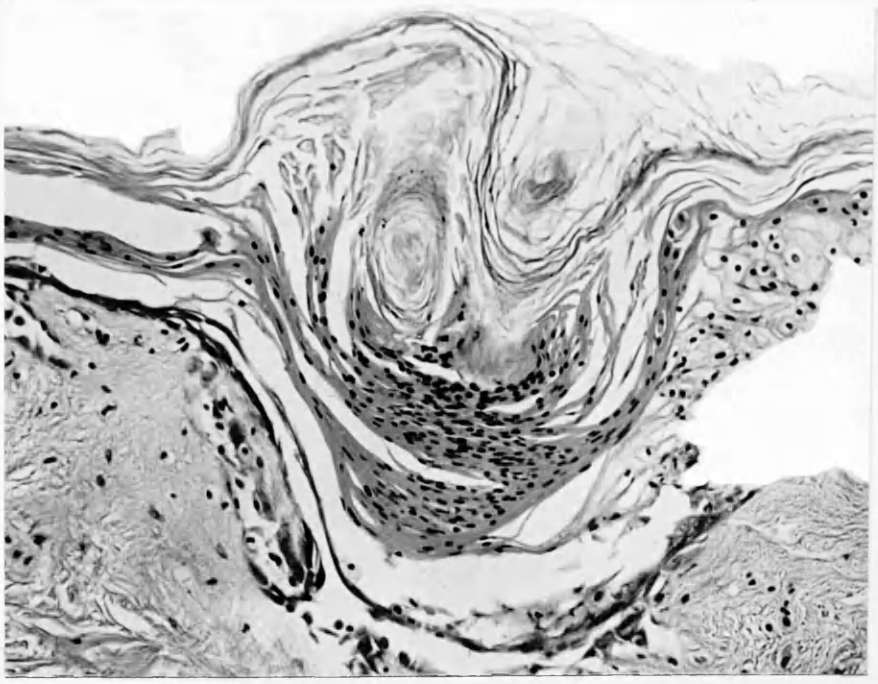


Fig. 75. Adult human skin cultured for 4 days with oestradiol; a dense area of parakeratosis. Haematoxylin and eosin x 225.

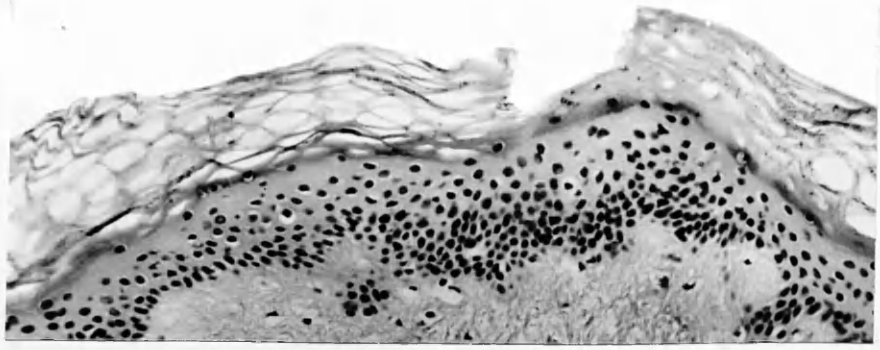


Fig. 76. Human adult skin after 4 days in culture with vitamin A; basal cell proliferation.
Haematoxylin and eosin x 225.

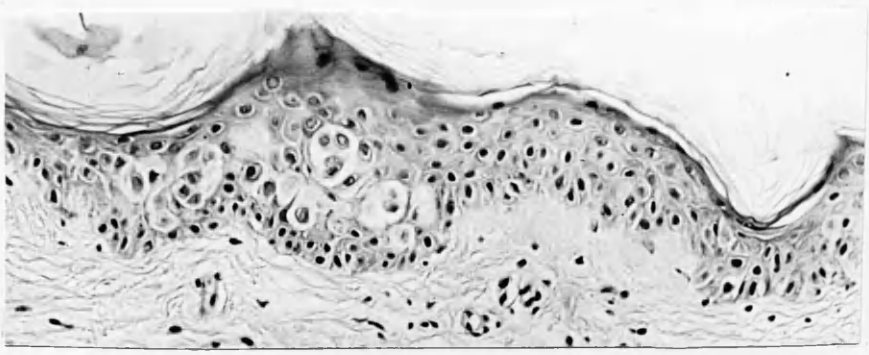


Fig. 77. Human adult skin after 6 days in culture with vitamin A; vacuolation of the squamous cells.
Haematoxylin and eosin x 225.

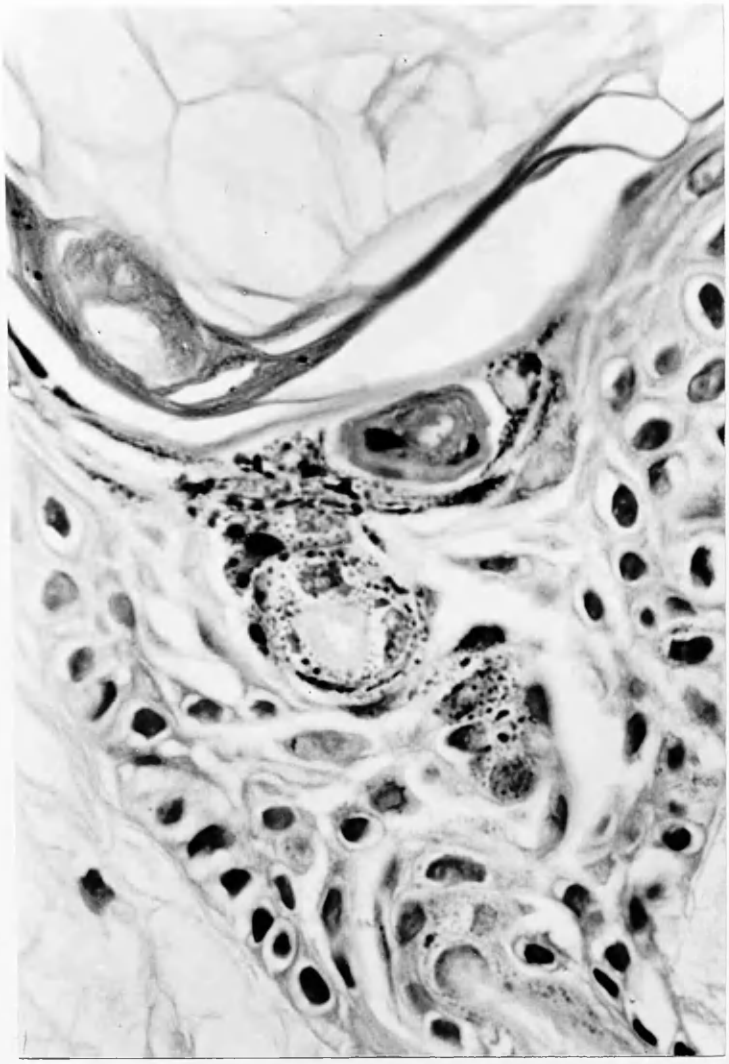


Fig. 78. Human adult skin after 6 days in culture with vitamin A; an isolated area of keratohyalin granules.
Haematoxylin and eosin x 950.

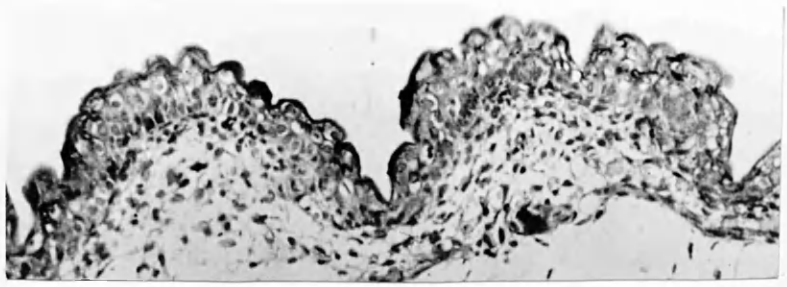


Fig. 79. 12 day foetal mouse skin; keratin is not present.
Haematoxylin and eosin x 225.

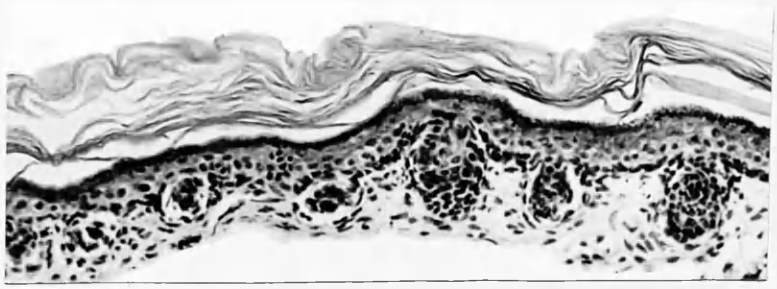


Fig. 80. 18 day foetal mouse skin; there is a prominent granular layer and well formed keratin.
Haematoxylin and eosin x 225.

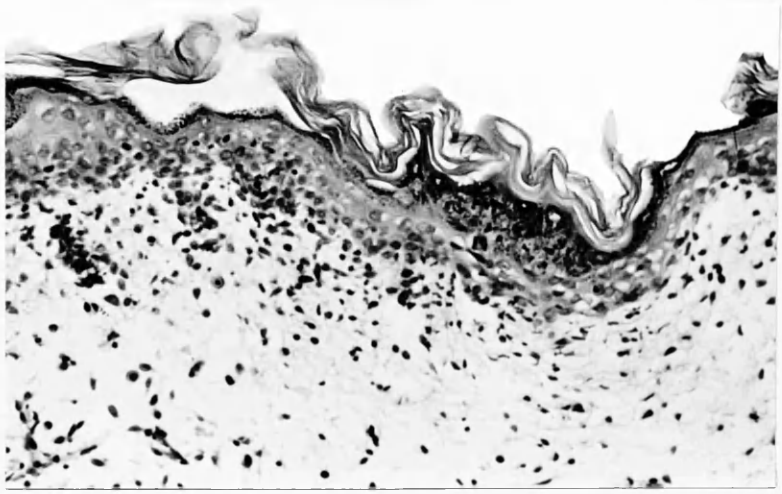


Fig. 81. Keratinised foetal mouse skin after 2 days in culture; there is epithelial proliferation and detachment of the keratin layer. Haematoxylin and eosin x 225.

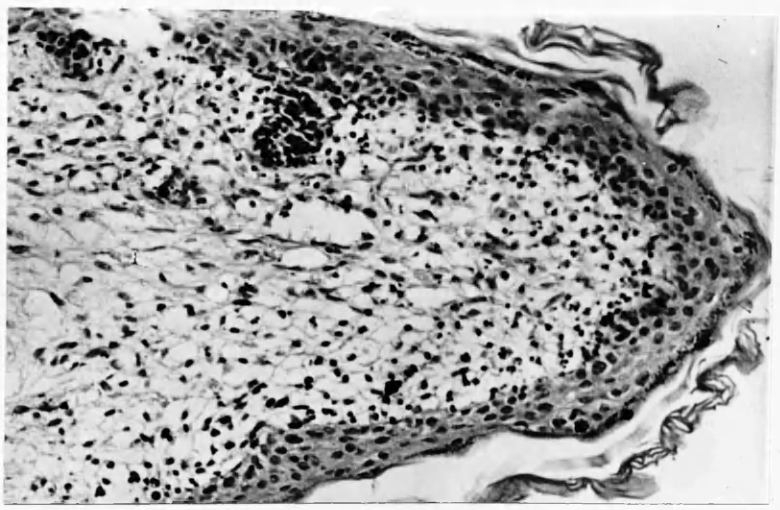


Fig. 82. Keratinised foetal mouse skin after 2 days in culture; epithelial cells are growing along the undersurface.
Haematoxylin and eosin x 225.

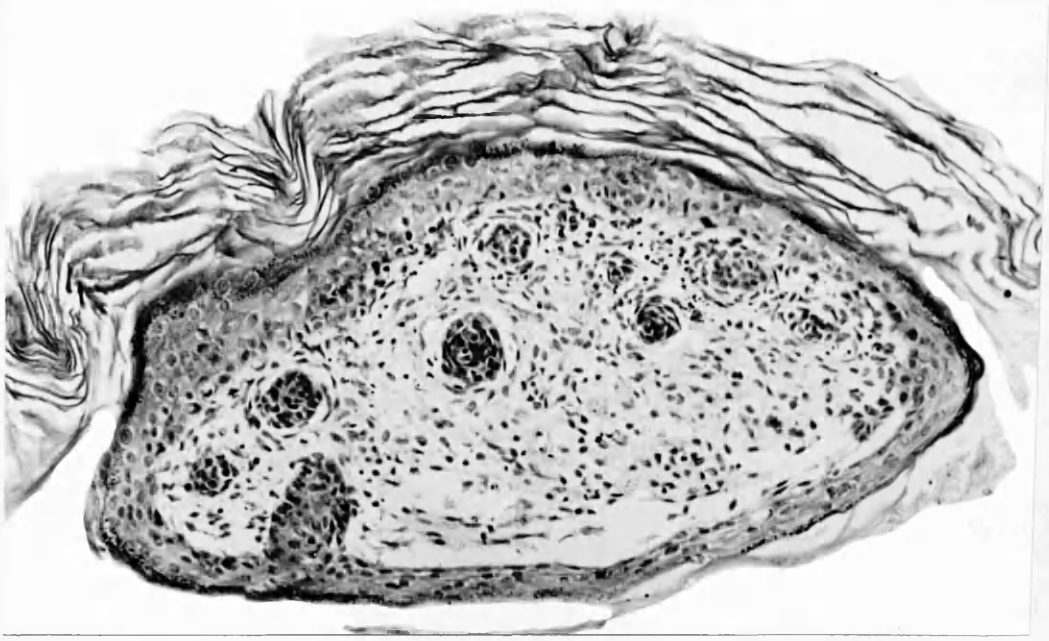


Fig. 83. Keratinised foetal mouse skin after 4 days in culture; epithelium surrounds the culture and there is abundant keratin. Haematoxylin and eosin x 225.

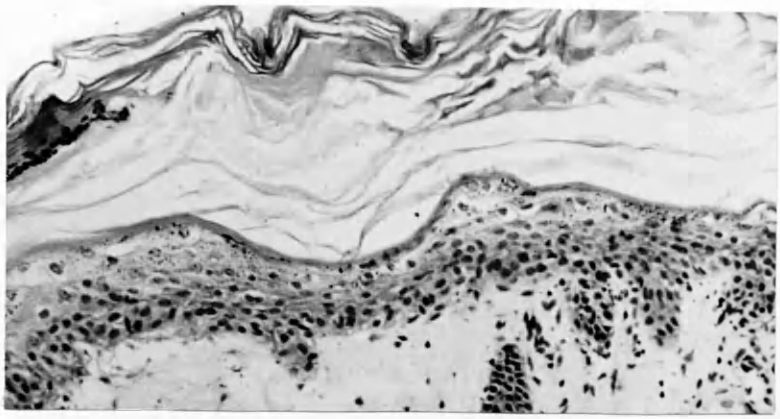


Fig. 84. Keratinised foetal mouse skin after 6 days in culture; actively growing squamous cells are covered by a broad layer of keratin.
Haematoxylin and eosin x 225.

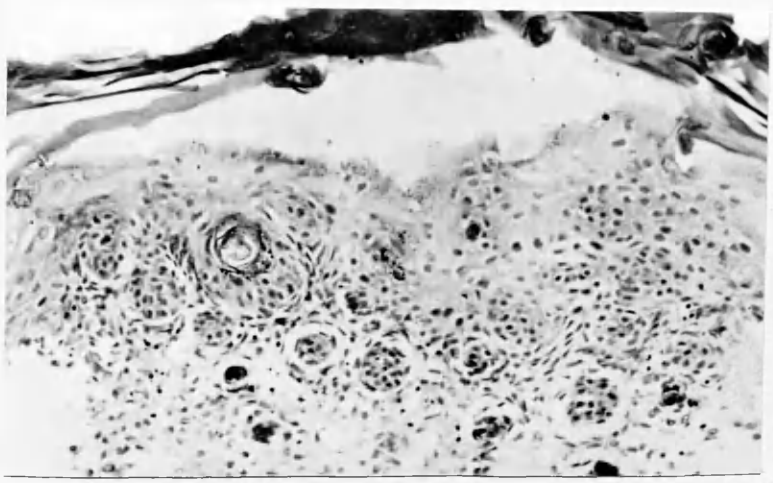


Fig. 85. Keratinised foetal mouse skin after 8 days in culture; the squamous cells are now pleomorphic and the superficial keratin loosely attached.
Haematoxylin and eosin x 225.

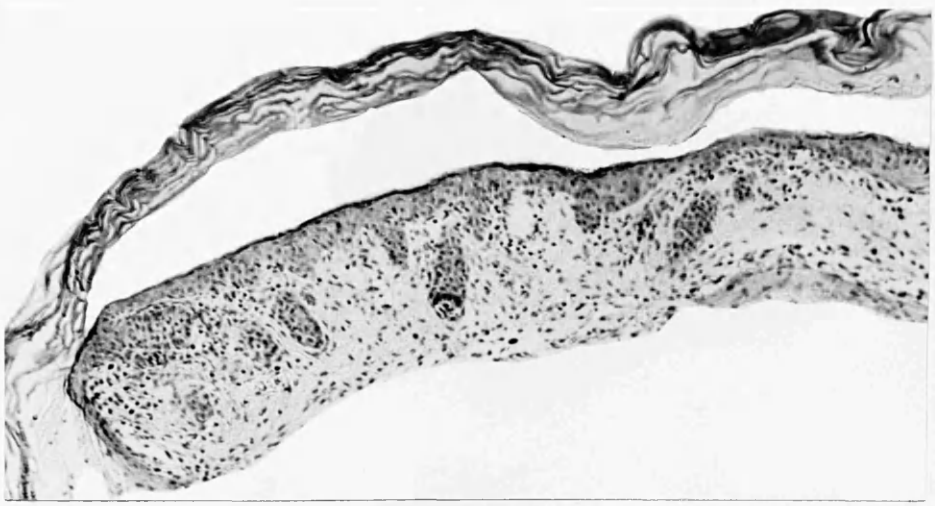


Fig. 86. Keratinised foetal mouse skin cultured with oestradiol for 2 days. The pre-formed keratin is largely detached.
Haematoxylin and eosin x 150.

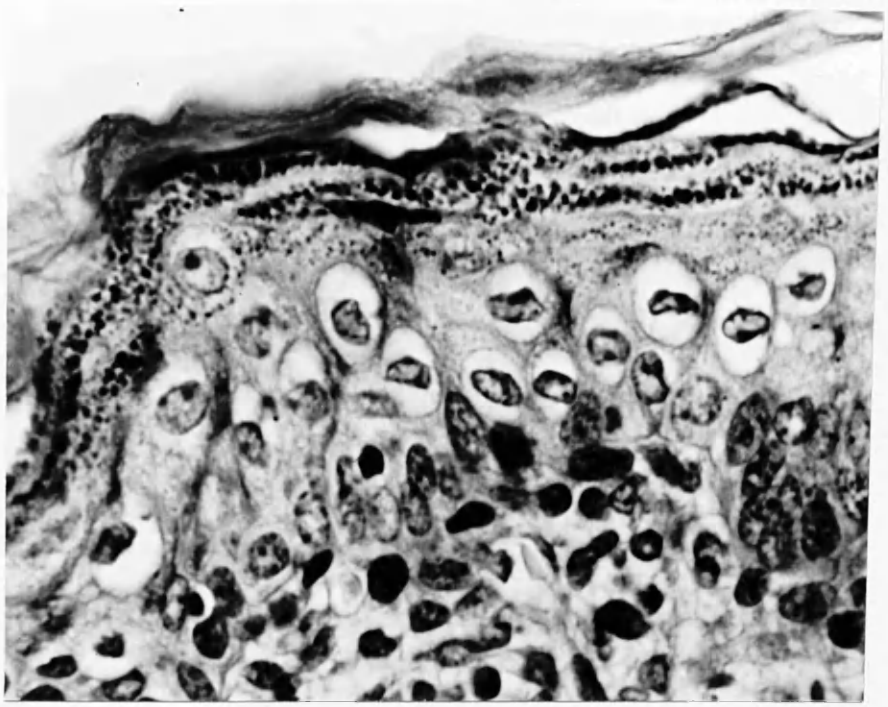


Fig. 87. Keratinised foetal mouse skin cultured with oestradiol for 2 days; the squamous cells are large and extend into the underlying dermis.
Haematoxylin and eosin x 950.

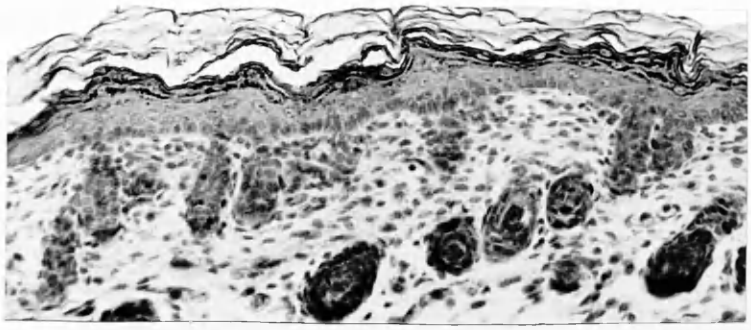


Fig. 88. Non-keratinised foetal mouse skin after culture for 4 days; a dense layer of keratin has now been formed.
Haematoxylin and eosin x 225.

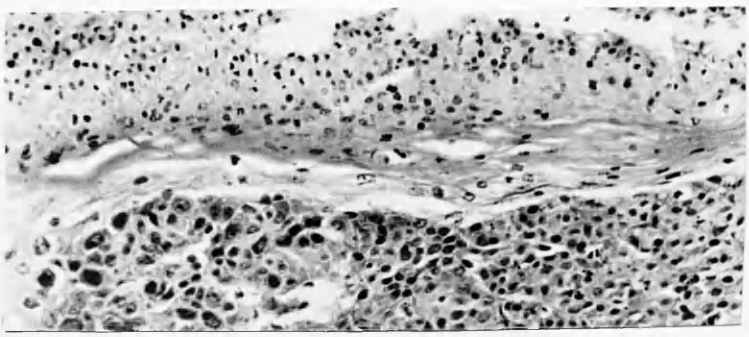


Fig. 89. Non-keratinised foetal mouse skin after culture for 6 days with oestradiol. The squamous cells are pleomorphic.
Haematoxylin and eosin x 225.

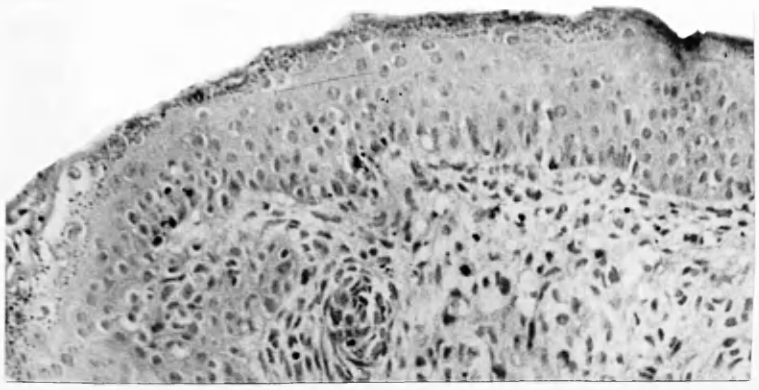


Fig. 90. Non-keratinised foetal mouse skin after culture for 4 days with vitamin A; the cells are squamous and there is a very thin superficial layer of keratin.
Haematoxylin and eosin x 225.

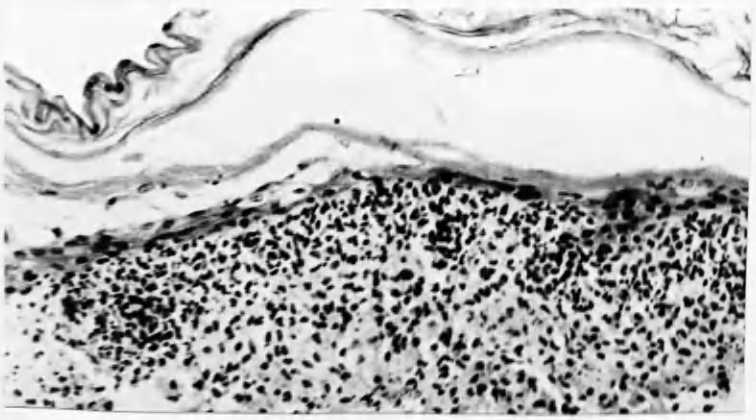


Fig. 91. Non-keratinised foetal mouse skin after culture for 8 days with vitamin A; the epithelium now consists of flattened epithelial cells. Keratin has been formed.
Haematoxylin and eosin x 225.

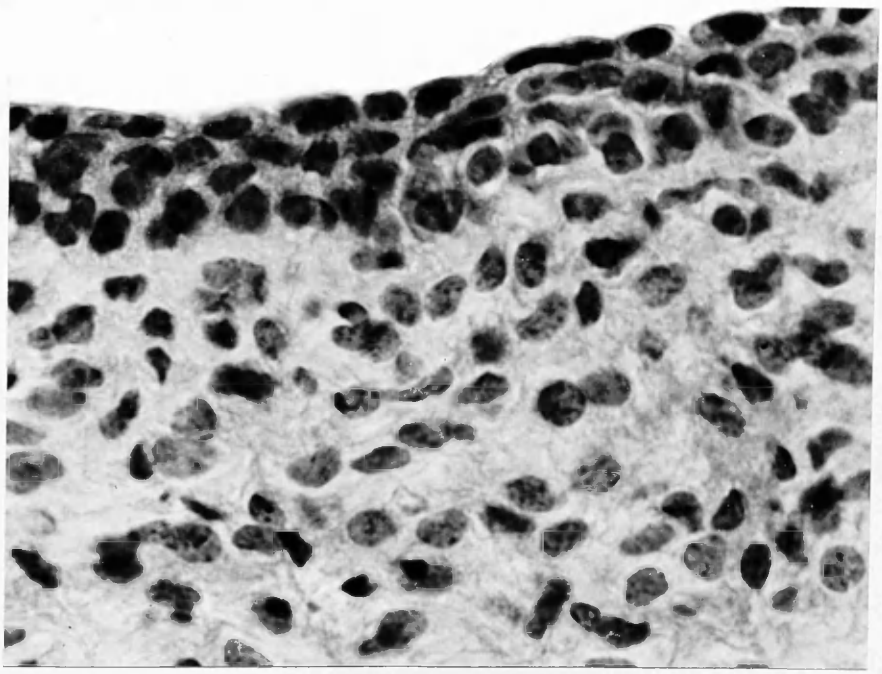


Fig. 92. Normal infantile mouse vagina; it is composed of large cuboidal cells.
Haematoxylin and eosin x 950.

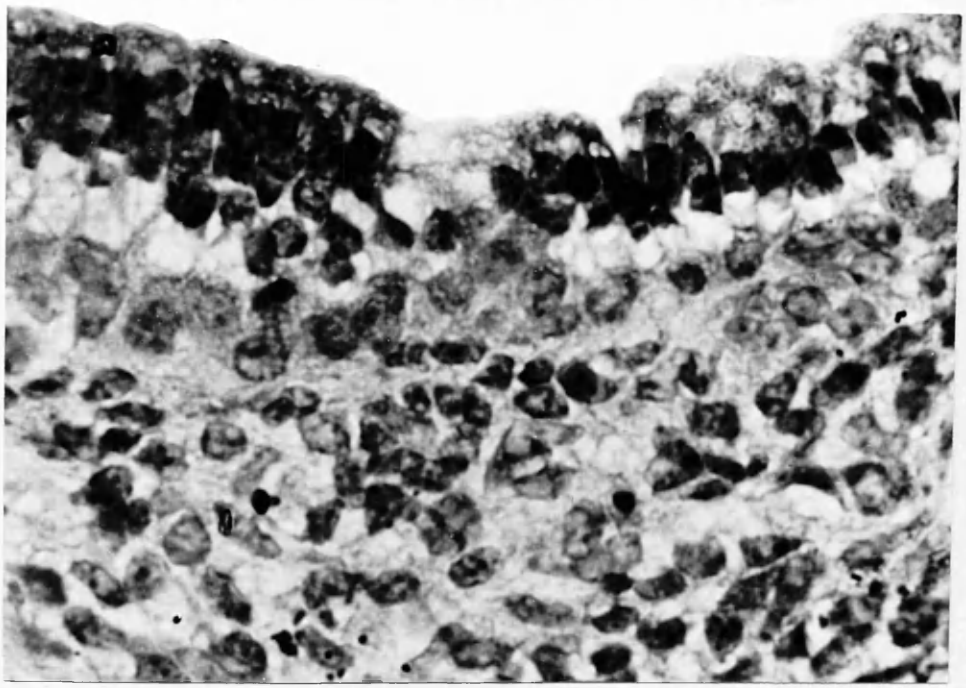


Fig. 93. Infantile mouse vagina after 2 days in culture; the lower layers now show some squamous differentiation. Haematoxylin and eosin x 950.

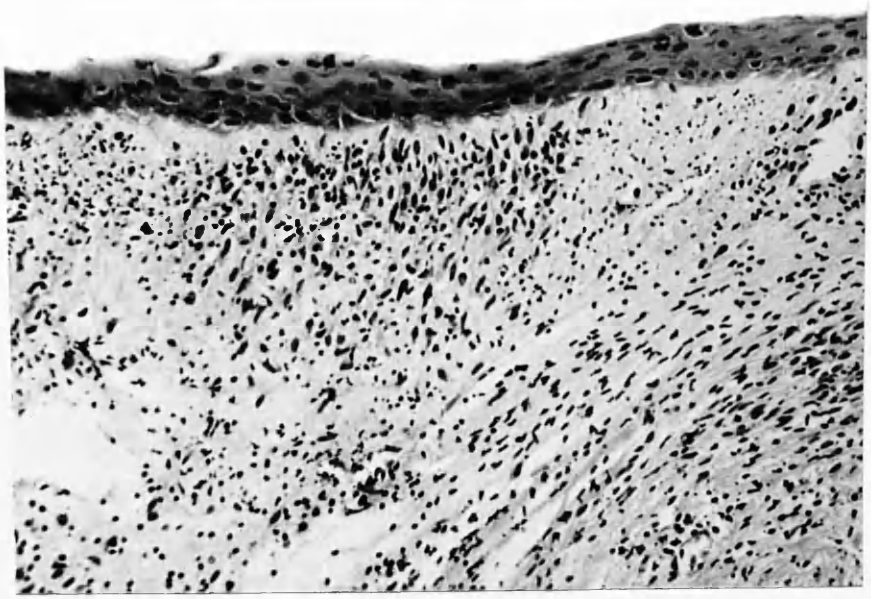


Fig. 94. Infantile mouse vagina after 4 days in culture; the epithelium is distinctly squamous in type. Haematoxylin and eosin x 225.

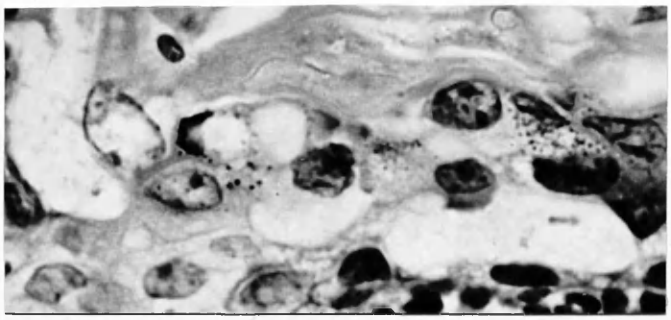


Fig. 95. Infantile mouse vagina after 4 days in culture; scanty keratohyalin granules are present.
Haematoxylin and eosin x 950.

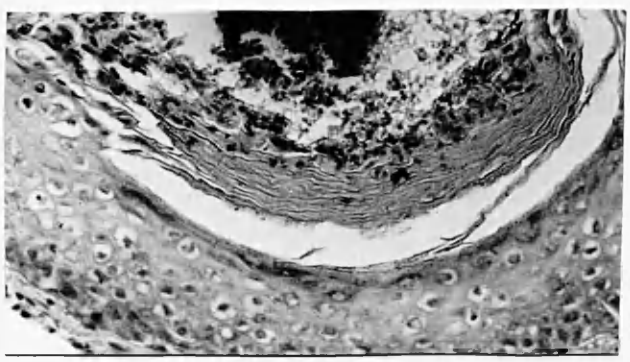


Fig. 96. Infantile mouse vagina after 8 days in culture; it is covered by a dense layer of keratin on top of which are degenerate cuboidal cells.
Haematoxylin and eosin x 225.

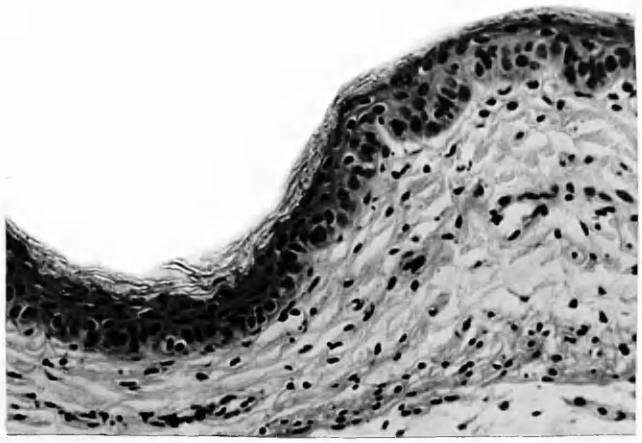


Fig. 97. Infantile mouse vagina after 4 days in culture with oestradiol; a well formed keratin layer is present. Haematoxylin and eosin x 225.

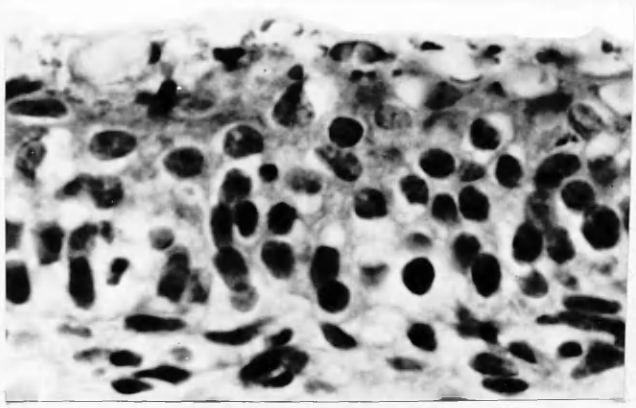


Fig. 98. Infantile mouse vagina after 4 days in culture with testosterone; the cells show slight squamous development. Haematoxylin and eosin x 950.

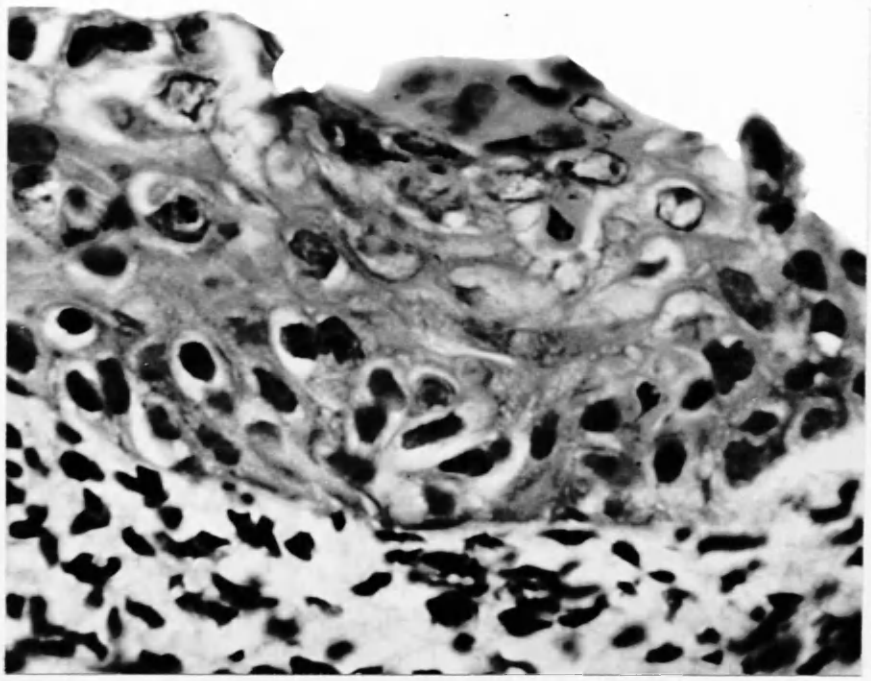


Fig. 99. Infantile mouse vagina in culture for 6 days with testosterone; the cells are now more distinctly squamous in type.
Haematoxylin and eosin x 950.

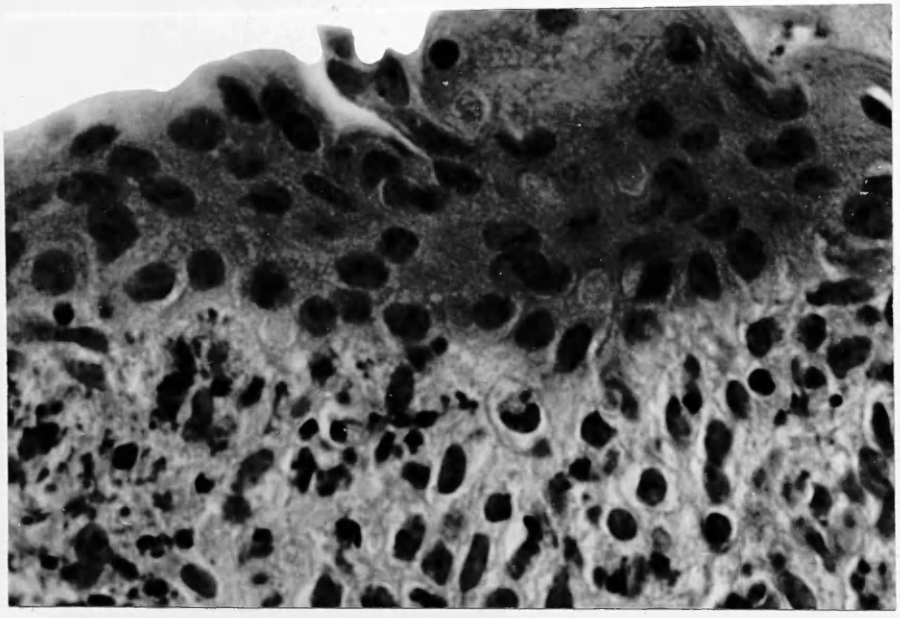


Fig. 100. Infantile mouse vagina after 6 days in culture with vitamin A; the cells show some squamous development. Haematoxylin and eosin x 950.

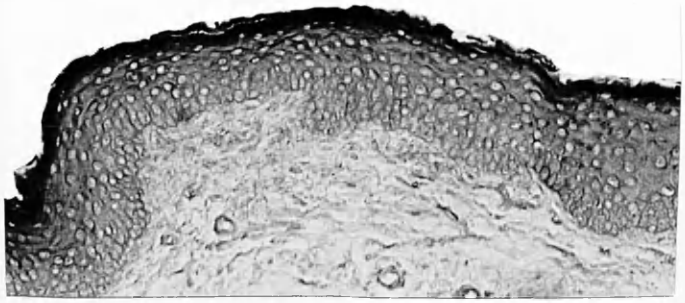


Fig. 101. Adult oestrus vagina; after 2 days in culture, an intense reaction for sulphydryl is present in the superficial squamous cells. Dihydroxy-dinapthyl-disulphide x 135.

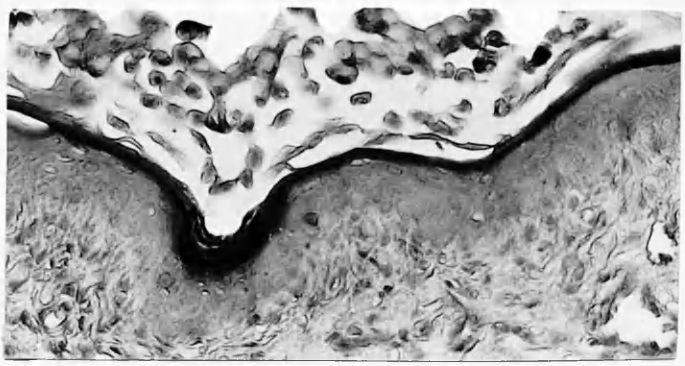


Fig. 102. Adult oestrus vagina after 4 days in culture; there is an intense reaction for disulphide in the keratin layer. Dihydroxy-dinapthyl-disulphide x 135.

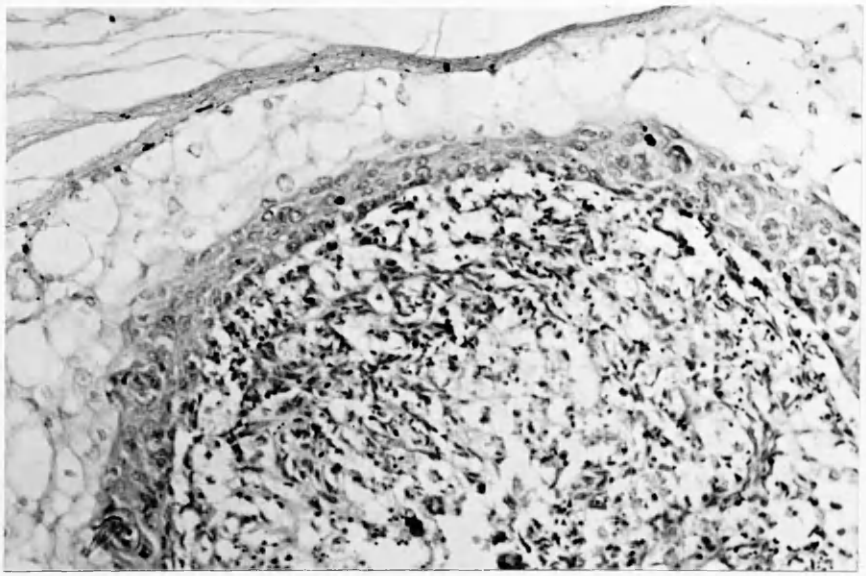


Fig. 103. Adult oestrus vagina after 6 days in culture; there is a superficial layer of keratin in which there are degenerating nuclei.
Haematoxylin and eosin x 225.

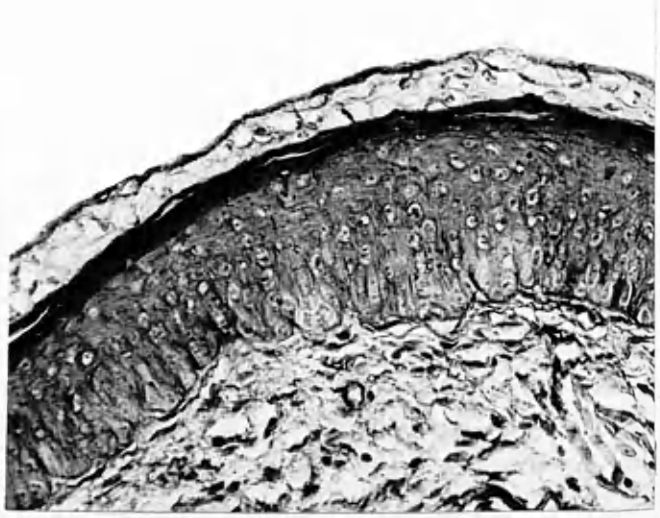


Fig. 104. Adult di-oestrus vagina after 2 days in culture; an intense reaction for sulphydryl in the superifical squamous cells. Dihydroxy-dinapthyl-disulphide x 225.

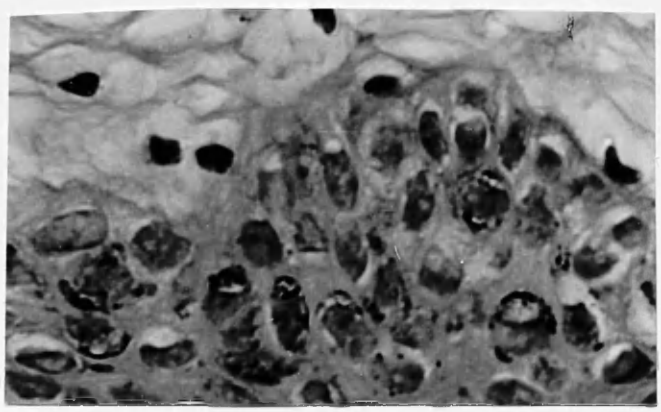


Fig. 105. Adult di-oestrus vagina after 2 days in culture; the squamous cells contain abundant glycogen.
Periodic acid-Schiff x 950.

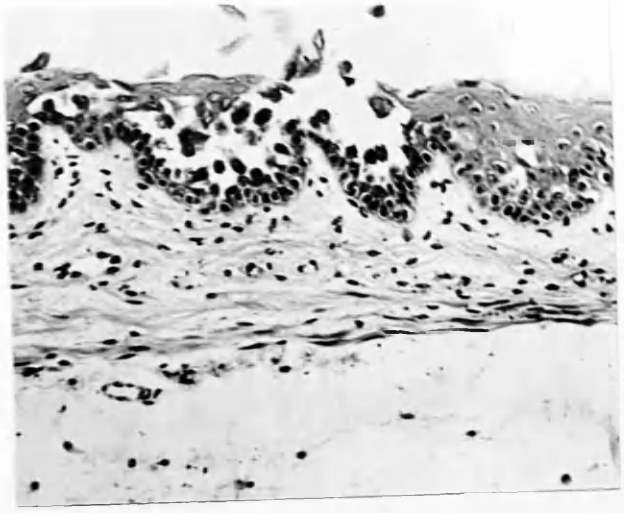


Fig. 106. Adult oestrus vagina after 1 day in culture with testosterone; desquamation of the squamous cell layer. Haematoxylin and eosin x 105.

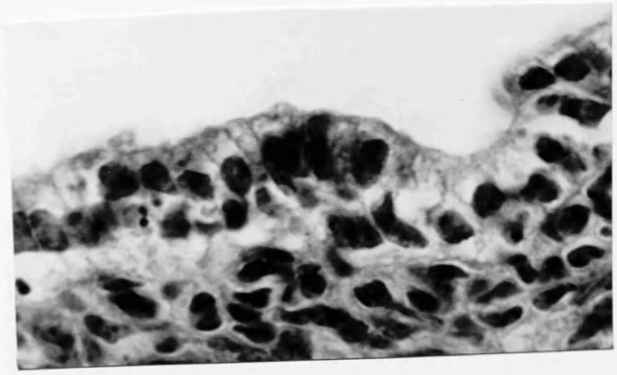


Fig. 107. Adult oestrus vagina after 4 days in culture with testosterone; the cells are now cuboidal in type. Haematoxylin and eosin x 950.

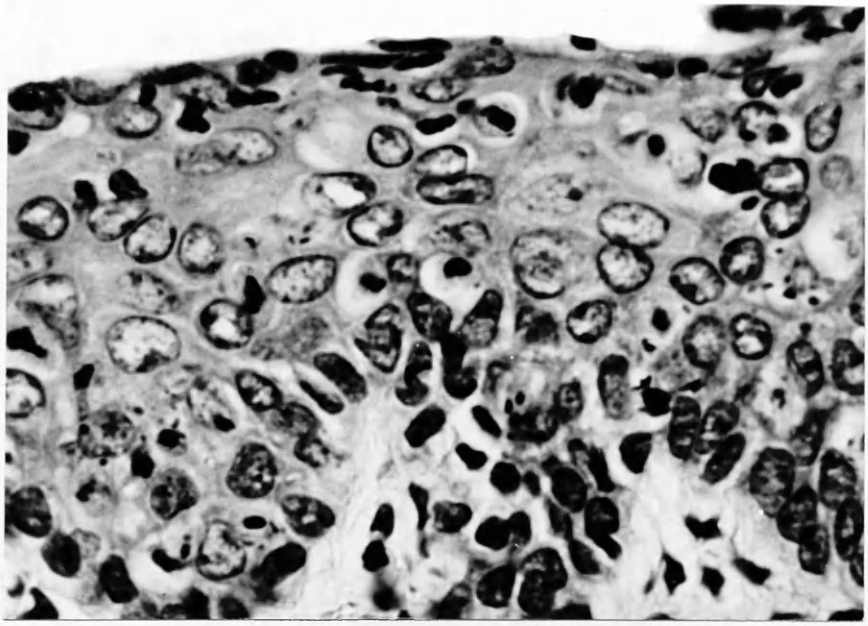


Fig. 108. Adult di-oestrus vagina after 4 days in culture with testosterone; the cells are squamous in type and there is superficial parakeratosis. Haematoxylin and eosin x 950.

Fig. 109. Adult oestrus vagina after 1 day in culture with progesterone; separation of the squamous cell layer. Haematoxylin and eosin x 225.

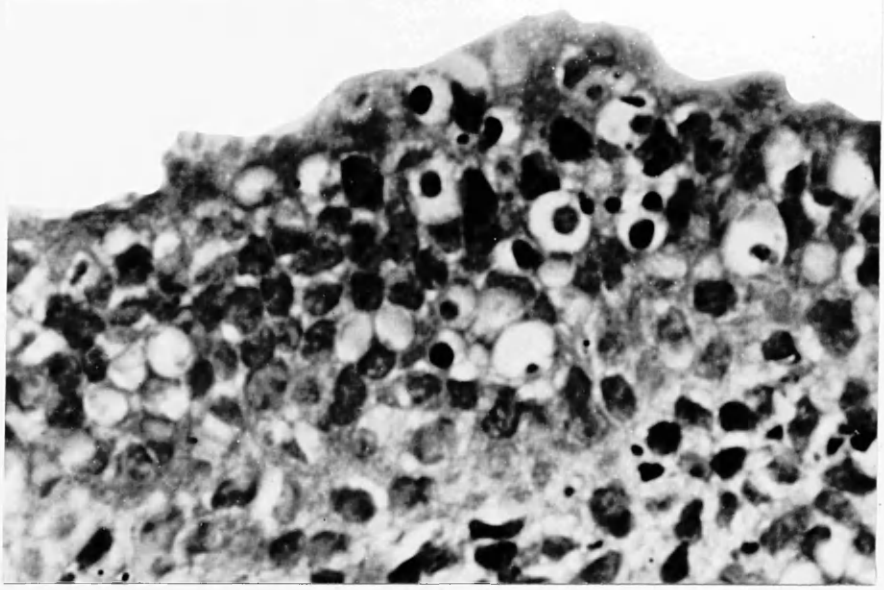


Fig. 110. Adult di-oestrus vagina after 4 days in culture with progesterone; many cells are vacuolated and contain pyknotic nuclei.
Haematoxylin and eosin x 950.

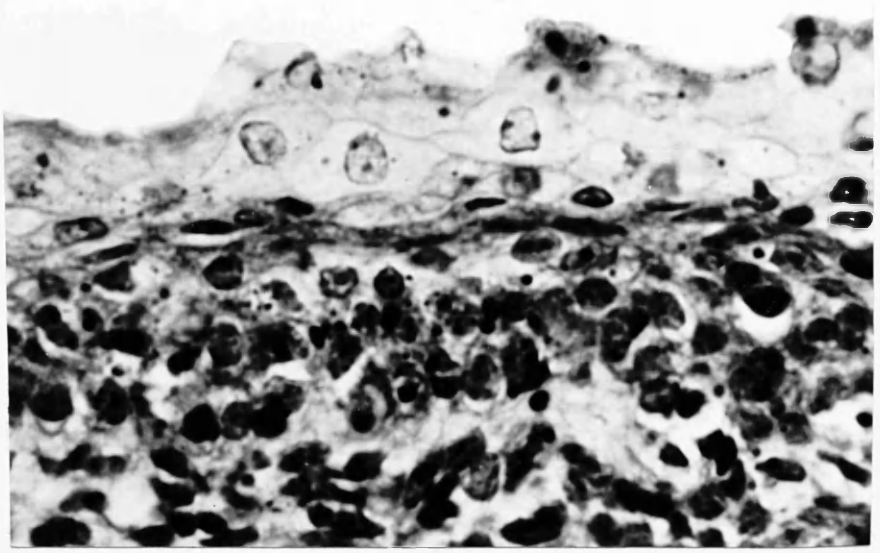


Fig. 111. Adult di-oestrus vagina after 6 days in culture with progesterone; the superficial cells are squamous in type.
Haematoxylin and eosin x 950.

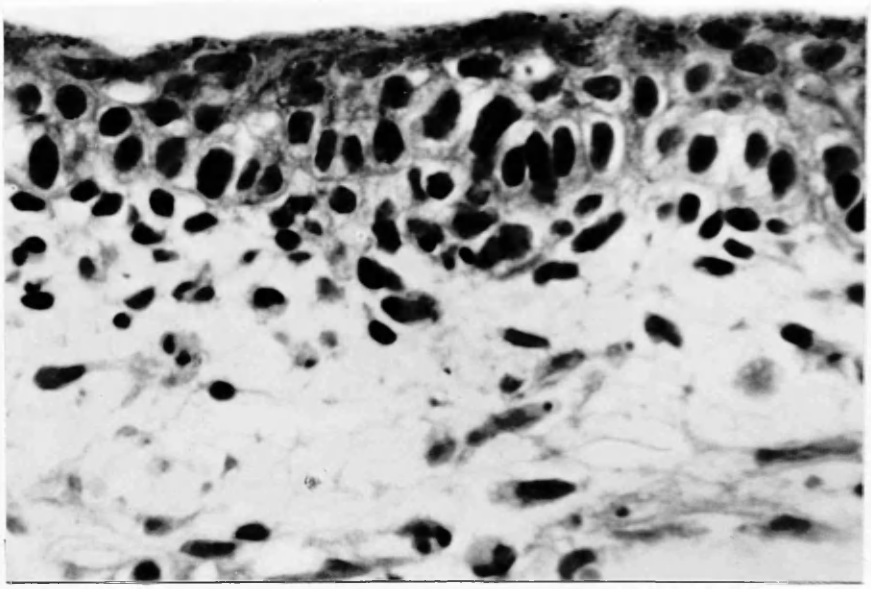


Fig. 112. Adult oestrus vagina after 4 days in Vitamin A; the cells are still mainly cuboidal and there are superficial keratohyalin granules.
Haematoxylin and eosin x 950.

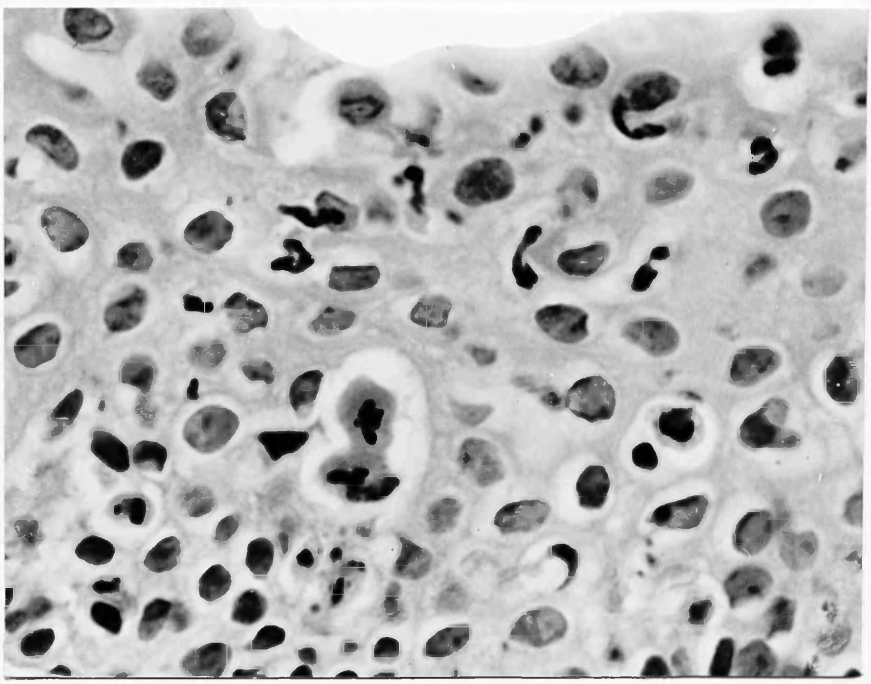


Fig. 113. Adult oestrus vagina after 6 days in Vitamin A; the cells are squamous in type and many are vacuolated. Haematoxylin and eosin x 950.

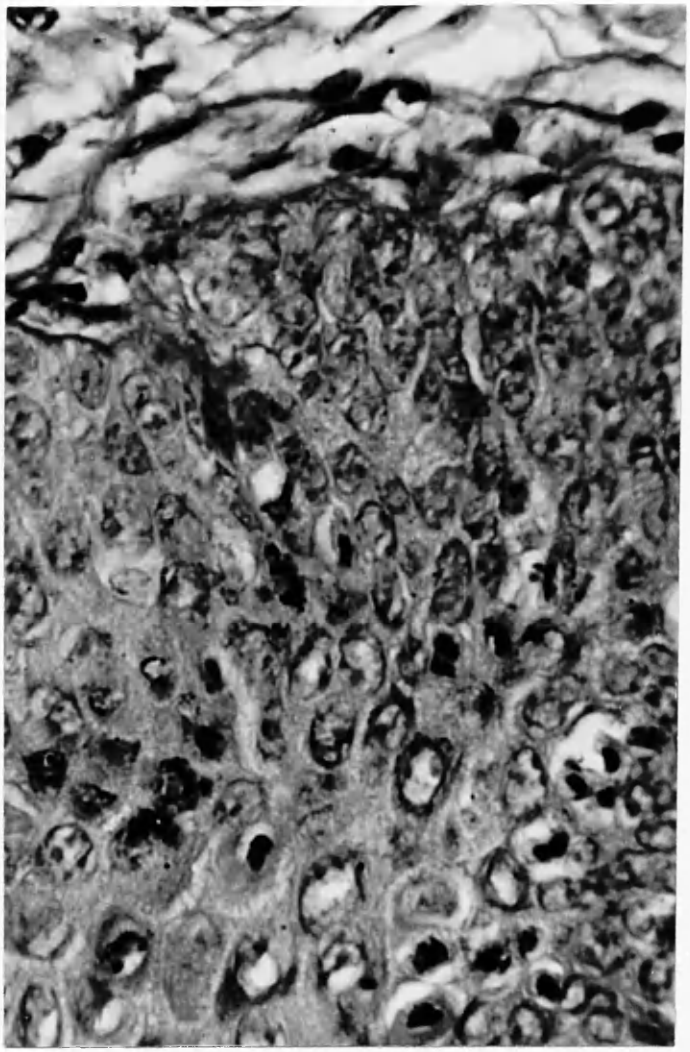


Fig. 114. Adult oestrus vagina after 8 days in culture with Vitamin A; the squamous cells contain abundant glycogen.
Periodic acid-Schiff x 950.

TECHNICAL APPENDIX

TECHNICAL METHODS

FIXATION

Human skin lesions were initially divided into three pieces and one part fixed for 24 hours in Formol-Zenker, Formol-corrosive or 10% neutral aqueous Formalin. The most satisfactory results were obtained with neutral formalin and as the tissues were generally not more than 4 mm. in thickness 12 hours fixation was routinely used. A similar period in this fixative was used for the cultured tissues. Where other fixatives were used they are given in the section on staining methods.

PROCESSING PROCEDURE

Paraffin sections cut at 6 μ were used routinely. The following method gave good results. The tendency for skins to become brittle, particularly those with hyperkeratosis, was minimised by employing rather shorter periods of dehydration than is usual and by clearing in cedar wood oil.

70% alcohol	½ hour
90% alcohol	½ hour
Absolute spirit	1 hour
Absolute spirit	1 hour
Absolute alcohol	1 hour
Cedar wood oil	overnight

Rinse excess with xylol.

Wax bath at 56°C for 2 hours - twice.

Embed in fresh wax.

STAINING METHODS

As described in the text three staining methods proved of value in the present study. These are Haematoxylin and Eosin, the Dihydroxy-dinapthyl-disulphide method for sulphydryl and disulphide and the periodic acid-Schiff method for glycogen. These methods will be described in detail. The other methods employed contributed relatively little and will only be described where their method of use differs from that given in standard textbooks.

Haematoxylin and Eosin: Harris's Haematoxylin (Carleton & Drury, 1957) was generally used and proved most satisfactory.

1. Bring paraffin sections to water.
2. Haematoxylin 3 minutes.
3. Wash with tap water until blue (approximately 2 minutes).
4. Decolourise in 1% acid-alcohol.
5. Wash in tap water until blue (approximately 3 minutes).
6. Counterstain with 1% watery eosin - 10 seconds.
7. Rinse with tap water.
8. Dehydrate with (a) 70% alcohol, (b) absolute alcohol.
9. Clear with xylol.
10. Mount in 'D.P.X.'

The Dihydroxy-Dinapthyl-Disulphide Method for SH and SS: (Barrnett & Seligman, 1952 and 1954). Although the authors recommend fixation with 1% trichloracetic acid in 70% alcohol, equally good results were obtained with formalin fixed material.

Sulphydryl:

1. Take sections to water.
2. Incubate slides in a coplin jar for 1 hour at 50°C in a solution prepared by mixing 35 ml. 0.1 M Michaelis barbital buffer, (pH 8.5) and 15 ml. absolute ethyl alcohol containing 25 mg. 2.2 di-hydroxy, 6.6 di-napthyl-disulphide (1). The reagent is almost completely soluble in above alcohol buffer mixture.
3. Cool for 10 minutes at room temperature.
4. Rinse briefly in distilled water.
5. Wash for 10 minutes in two changes of distilled water acidified to pH 4-4.5 with acetic acid.
6. Pass through a graded series of alcohols and wash in absolute ether for 5 minutes.
7. Dehydrate and rinse in distilled water.
8. Stain for 2 minutes at room temperature in the following freshly prepared mixture - 50 mg. tetrazotized di-orthoanisidine in 50 ml. 0.1 M Sorensen phosphate buffer, pH 7.4.
9. Wash in running tap water.
10. Mount with glycerogel.

Although the original authors state that step No. 6 should be carried out rapidly, it has been found that this should not be done too hurriedly - approximately 10 seconds in each reagent.

Disulphide:

1. Cover tissue with 2% celloidin.
2. Incubate 20-24 hours at 37°C in 0.1 M N-ethyl-maleimide.
3. Incubate in 10% pot. cyanide for 2 hours at 37°C.
4. Wash very thoroughly in running water.
5. Stain with DDD as above.

Periodic acid-Schiff Method:

1. Bring paraffin sections to water.
2. Immerse in periodic acid solution - 5 minutes.
3. Rinse in distilled water.
4. Pour on Schiff's reagent and leave for 20 minutes.
5. Pour on 3 changes of sulphurous acid - 3 minutes in each.
6. Wash in running water for 3 minutes.
7. Counterstain with haematoxylin for 1 minute and blue with Scott's tap water substitute.
8. Dehydrate, clear and mount in DPX.

The Schiff's reagent and sulphurous acid solution are prepared as described by Carleton and Drury (1957).

Use of diastase:

1. Bring paraffin sections to water.
2. Cover with a 1% solution of diastase (Light & Co.) in distilled water for 30 minutes at room temperature.
3. Stain sections by periodic acid-Schiff method as above.

Feulgen Reaction for DNA: The method used is that of Feulgen and Rossenbeck (1924) modified by Pearse (1960). The optimum time of hydrolysis was found to be 10 minutes at 60°C.

The Methyl Green-Pyronin Method for RNA: (Brachet, 1942). As suggested by Pearse (1960), orange b was omitted from solution B. Fresh solutions were prepared weekly and unsatisfactory batches, as judged by control sections, discarded.

Ribonuclease extraction was carried out at 37°C for one hour with a 0.5% aqueous solution of the enzyme.

Acid Phosphatase: A modification of Gomori's original method by

Holt (1959) was used. Frozen sections were prepared from tissues fixed for 24 hours in cold formol-calcium solution (4% w/v formaldehyde and 1% CaCl) and post fixed for 7 days in cold hypertonic gum sucrose solution.

Alkaline Phosphatase: The Azo coupling dye technique as described by Pearse (1960) was used on frozen sections from tissues fixed in cold neutral formalin for 15 hours.

ANIMAL METHODS

Stock animals: The tumours are carried in female LAF 1 mice. These are first generation hybrids from the mating of female C57 Leaden/Jax and male A/Heston Jax. The parent stocks are obtained by the brother and sister mating of pedigree strains originally obtained from Jackson Memorial Laboratory, Bar Harbor, Maine.

Tumour transplantation: The animals are killed by cervical dislocation and the tumours dissected with aseptic precautions. They generally measure 1 cm. in diameter. After washing in Medium 199 they are minced with scissors and medium added to make an approximate 20% suspension. 0.05 ml. of this fluid is injected into the muscles of the right thigh using a tuberculin syringe and a number 1 needle.

Vaginal smears: The method is that devised by Dr. Beatrice Pullinger. A drop of water is placed on a slide from a small platinum loop. The vagina is stroked gently with the loop and the adherent cells mixed with the drop of water and smeared on the slide. Between smearing each animal the loop is sterilised by heating in a gas jet. Six smears can be placed on each slide. They are fixed by immersion in formol-corrosive for 2 minutes and stained with 1% aqueous methylene blue.

TISSUE CULTURE METHODS

A. PLASMA CLOT CULTURES

1½ x 3/8" glass coverslips (Chance No. 2) are lightly coated with chick plasma (Difco T.C. 0483) and left for 15 minutes. Pieces of tissue not more than 2 mm. square are immersed briefly in chick embryo extract (Difco T.C. 0355 diluted to EE 20). The fragments are then placed on the coated slide and left for 20 minutes until a firm gel has formed. The coverslips are then placed in a 6 x 5/8" test tube and medium added. The tubes are sealed with silicone stoppers and incubated stationary at 37°C, and turned twice daily. For examination the coverslips are removed and the cultures fixed in alcohol and stained by dilute Giemsa.

The plasma clot cultures in watch glasses are prepared in a similar way; 0.4 ml. each of embryo extract and chick plasma being used to form the clot. Tissues are removed every second day, washed in saline and placed on fresh clot. For examination the entire clot is fixed in neutral aqueous formalin and paraffin sections prepared.

B. ORGAN CULTURES

(i) Materials:

1. 1½ x 1½" flat watch glasses (Baird & Tatlock Glass block D6/612). 2. Cellulose acetate sponge (commercial foam plastic).

3. Lens Paper (Reeve-Angel, New York, No. 43650). 4. Culture chamber.

(ii) Procedure: Tissues are cut into, at largest, 2 mm. square pieces with scalpels (straight blades; Swann-Morton, No. 11, have been found to be most satisfactory). For the dissection of small tissues a binocular dissecting microscope with a magnification of x 10 is used. The pieces of tissue are washed in medium 199 to remove any blood or fragmented tissue. They are then placed on lens paper which rests on filter paper moistened with medium. The lens paper is then laid carefully on top of the sponge which is in the centre of the culture dish partially filled with medium. Further medium is added until the top of the sponge is reached and the tissue partially covered by culture medium. The dishes are then transferred to the culture chamber which is previously heated to 37°C and flushed with 5% CO_2 in oxygen. The chamber is generally flushed twice daily and unless indicated in the text, e.g., vitamin A cultures, the medium is changed daily. For examination the lens paper with the tissue are transferred to neutral formalin and paraffin sections prepared.

(iii) Construction of the tissue culture chamber: The material used is clear acrylic plastic (I.C.I. Perspex) and the parts joined with a cement, made by dissolving pieces of 'Perspex' in Ethylene Chloride until a syrupy solution is obtained. The chamber consists of a rectangular box with the internal measurements, 10_4" long, 8"

broad and 10" deep, the sides being $\frac{1}{4}$" thick. The flange at the top of the box is made in one piece and cemented to the box. Each of the sides of this part is $1\frac{1}{4}$" broad, thus allowing 1 inch to project beyond the outside of the box. 14 $\frac{1}{4}$" clearance holes are drilled in the flange to take the bolts which fasten down the cover, the holes being arranged 5 on each of the long sides and 4 on each of the short sides.

The cover is made from $\frac{1}{4}$" perspex and sealed with a 1/16" thick rubber joint. 14, $\frac{1}{4}$" x 1" bolts with wing nuts secure the cover. Inlet and outlet holes are drilled at the bottom and top of opposite sides of the chamber, and are tapped to take brass connections for rubber tubing.

Shelves, $\frac{1}{4}$ x $\frac{1}{4}$ x 8" are attached to both of the short sides at a height of 2" from the top. These carry a large tray. To increase the number of cultures in the chamber a rack was made to fit between the shelves. This carries four smaller trays $1\frac{3}{4}$" apart. One side of the rack is left open to allow the trays to slide out. The trays are made from 1/8" thick strips attached to form squares, into which the culture dishes are placed. Holes are drilled in these strips to facilitate the circulation of gas. The trays carry 12 dishes each, giving a total capacity of 60 dishes.

CARE OF GLASSWARE

Culture dishes:

1. Place in hot soapy water - 12 hours.

2. Wash thoroughly.
3. Rinse in running tap water.
4. Place in three changes of distilled water.
5. Inspect - if not clean return to stage 2.
6. Dry.
7. Rubber capped bottles are autoclaved - dishes and petri dishes put into containers and sterilised at 180°C for 1 hour.

Pipettes:

1. Clean in 10% Potassium dichromate solution.
2. Wash in automatic pipette washer overnight.
3. Place in two changes of distilled water.
4. Dry.
5. Inspect and place into containers and sterilise at 18°C for 1 hour.

Preparation of sponges and lens paper: Sponges and paper are cut into strips (1 x ½ cm.).

1. Immerse in distilled water for ½ hour.
2. Immerse in methylated spirit for ½ hour.
3. Boil in absolute alcohol.
4. Immerse in ether for 1½ hours.
5. Immerse in absolute alcohol 1 hour.
6. Immerse in methylated spirit 1 hour.
7. Immerse in distilled water 1½ hours.
8. Dry at 140°C in gas oven.
9. Sterilise at 5 lbs. pressure for 20 minutes.

Stock Solutions of the steroids and other substances added to the medium contained 5 mg./ml. and were stored at 4°C (except Vitamin A which was freshly prepared). For use they were diluted so that 0.01 ml. of the alcoholic solution was added to 1 ml. of medium.

REFERENCES

ADAMS, C.W.M., AND SLOPER, J.C.	1955.	Lancet, 1, 651.
ADAMS, C.W.M., AND SLOPER, J.C.	1956.	J. Endocr., 13, 221.
ALEXANDER, P.	1951.	Ann. N.Y. Acad. Sci., 53, 653.
ALLEN, E.	1922.	Amer. J. Anat., 30, 297.
ALLEN, E., FRANCIS, B.F., ROBERTSON, L.L., COLGATE, C.E., JOHNSTONE, C.G., DOISY, E.A., KOUNTZ, W.B., AND GIBSON, H.V.	1924.	Amer. J. Anat., 34, 133.
ALLGOWER, M., POMERAT, C.M., AND BLOCKER, T.G. JNR.	1952.	Ann. Surg., 135, 923.
ALLOITEAU, J.J., AND COURVOISIER, B.	1953.	C.R. Soc. Biol. (Paris), 147, 553.
ASTBURY, W.T.	1933a.	Tr. Faraday Soc., 29, 193.
ASTBURY, W.T.	1933b.	Fundamentals of Fibre Structure, 1st Ed., London.
ASTBURY, W.T.	1942.	J. Chem. Soc., p. 337.
ASTBURY, W.T., AND WOODS, H.J.	1934.	Phil. Trans. A., 232, 333.
BAHN, R., FURTH, J., ANDERSON, E., AND GADSDEN, E.	1957.	Amer. J. Path., 33, 1075.

BARRNETT, R.J., AND SELIGMAN, A.M.	1952.	Science, 116, 323.
BARRNETT, R.J., AND SELIGMAN, A.M.	1954.	J. Nat. Cancer Inst., 14, 769.
BENNETT, H.S.	1951.	Anat. Rec., 110, 231.
BERENBLUM, I., CHAIN, E., AND HEATLEY, N.G.	1940.	Amer. J. Cancer, 38, 367.
BERNARD, C.	1859.	C.R. Acad. Sci., 48, 673.
BEVERIDGE, W.I.B., AND BURNET, F.M.	1953.	M.R.C. Special Report, London, Series No. 256.
BIESELE, J.J.	1944.	Cancer Res., 4, 737.
BIGGERS, J.D., AND CLARINGBOLD, P.J.	1954.	J. Endocr., 11, 277.
BIGGERS, J.D., CLARING-BOLD, P.J., AND HARDY, M.H.	1956.	J. Physiol. 131, 497.
BIVINS, J.A.	1953.	J. invest. Derm., 20, 471.
BLANK, H., BUERK, M., AND WEIDMAN, F.	1951.	J. invest. Derm., 16, 19.
BLOCH, D.P., AND GODMAN, G.C.	1957.	J. exp. Med., 105, 161.
BLOCK, R.J.	1939.	J. biol. Chem., 128, 181.

BLOCK, R.J.	1951.	Ann. N.Y. Acad. Sci., 53, 608.
BLOOM, W.	1937.	Physiol. Rev., 17, 589.
BRACHET, J.	1942.	Arch. Biol. (Paris), 53, 207.
BRADFIELD, J.R.G.	1951.	Nature, 167, 40.
BRODY, I.	1959.	J. Ultrastructure Res., 3, 84.
BULLOUGH, H.F.	1942-44.	J. Endocr., 3, 280.
BULLOUGH, W.S.	1946.	Phil. Trans. B., 231, 165.
BULLOUGH, W.S.	1952.	Biol. Rev., 27, 133.
BULLOUGH, W.S.	1953.	Ciba Found. Collq. Endocr., 6, 278.
BULLOUGH, W.S.	1955.	In Vitamins and Hormones, New York, vol. 13, p. 261.
BURROWS, H.	1949.	In Biological actions of sex hormones, 2nd Ed., Cambridge, pp. 360-367.
CAMPBELL, J.G.	1949.	Brit. J. Cancer, 3, 72.
CARLETON, H.M., AND DRURY, R.A.B.	1957.	Histological Technique, London, pp. 100, and 145.
CARREL, A.	1924.	Physiol. Rev., 4, 1.
CHANG, R.S.	1957.	Special Report N.Y. Acad. Sci., 5, 315.

CHEN, J.M.	1954.	Exp. cell Res., 7, 518.
CHEVREMONT, M., AND FREDERIC, J.	1943.	Arch. Biol., 54, 589.
COHEN, A.I., AND FURTH, J.	1959.	Cancer Res., 19, 72.
COHN, E.J., AND EDSALL, J.T.	1943.	Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, 1st Ed., New York, p. 345.
COURRIER, R.	1942.	Ann. Endocr. (Paris), 3, 181.
COWDRY, E.V.	1940.	Amer. J. clin. Path., 10, 133.
D'AUNOY, R., AND EVANS, F.L.	1937.	J. Path. Bact., 44, 369.
DEMPSEY, E.W., AND WISLOCKI, G.B.	1944.	Endocrinology, 35, 409.
DREW, A.H.	1922.	Brit. J. exp. Path., 3, 20.
DREW, A.H.	1923.	Brit. J. exp. Path., 4, 46.
DUBREUILH, W.	1895.	Ann. Derm. Syph., (Paris), 6, 441.
DUX, C.	1941.	Ann. Endocr. (Paris), 2, 39.
EAGLE, H.	1958.	Ann. N.Y. Acad. Sci., 76, 545.
EDGREN, R.A.	1959.	Ann. N.Y. Acad. Sci., 83, 160.

EMMENS, C.W., AND BRADSHAW, T.E.T. 1939. J. Endocr., 1, 378.

EMMENS, C.W., AND LUDFORD, R.J. 1940. Nature, 145, 746.

FAWCETT, D.W. 1955. In An Introduction to Cell and Tissue Culture, 1st Ed., Minneapolis, p. 70.

FELL, H.B. 1951. Meth. med. Res., 4, 233.

FELL, H.B. 1953. Sci. Progr., 41, 212.

FELL, H.B., AND DANIELLI, J.F. 1943. J. exp. Path., 24, 196.

FELL, H.B., AND MELLANBY, E. 1953. J. Physiol., 119, 470.

FELSHER, Z. 1947. J. invest. Derm., 8, 123.

FERREIRA, A.E.M. 1941. J. Lab. clin. Med., 26, 1612.

FEULGEN, R., AND ROSSENBECK, H. 1924. Z. phys. Chem., 135, 203.

FISCHER, A. 1924. J. exp. Med., 39, 585.

FISCHER, E. 1953. Arch. Derm. Syph. (Berlin), 196, 256.

FISHER, I., AND GLICK, D. 1947. Proc. Soc. exp. Biol. (N.Y.), 66, 14.

FLESCH, P. 1952. J. invest. Derm., 19, 353.

FLESCH, P.	1953.	J. invest. Derm., 21, 421.
FLESCH, P.	1958.	J. invest. Derm., 31, 63.
FREUD, J.	1938.	Acta. brev. neerl. Physiol., 8, 127.
FURTH, J.	1955.	In Recent Prog. Hormone Res., New York, vol. 11, p. 221.
FURTH, J.	1957.	Cancer Res., 17, 454.
FURTH, J., BURNETT, W.T., AND GADSDEN, E.L.	1953.	Cancer Res., 13, 298.
FURTH, J., AND CLIFTON, K.	1957.	Cancer, 10, 842.
FURTH, J., UPTON, A.C., AND KIMBALL, A.W.	1959.	Radiat. Res. Suppl. 1, 243.
GARDNER, W.U.	1959.	Ann. N.Y. Acad. Sci., 83, 145.
GEY, G.O.	1956.	Harvey Lectures, Series L. 1954-1955, New York, p. 154.
GHERA, N., HARAN, PULLAR, P., AND FURTH, J.	1960.	Endocrinology, 66, 694.
GIROUD, A., BULLIARD, H., AND LEBLOND, C.P.	1934.	Bull. Histol. Tchn. micr., 11, 129.
GIROUD, A., AND CHAMPETIER, G.	1936.	Bull. Soc. Chim. Biol., 18, 656.

GIROUD, A., AND LEBLOND, C.P.	1951.	Ann. N.Y. Acad. Sci., 53, 613.
GOLDSWORTHY, N.E., AND MOPPETT, W.	1935.	J. Path. Bact., 41, 529.
GOODPASTURE, E.W., AND ANDERSON, K.	1940.	Arch. Path. (Chicago), 30, 212.
GOODPASTURE, E.W., AND ANDERSON, K.	1944.	Amer. J. Path., 20, 447.
GOODPASTURE, E.W., DOUGLAS, B., AND ANDERSON, K.	1938.	J. exp. Med., 68, 891.
GOODPASTURE, E.W., WOODRUFF, A.M., AND BUDDINGH, G.J.	1932.	Amer. J. Path., 8, 271.
GORBMAN, A.	1949.	Proc. Soc. exp. Biol., 71, 237.
GRIGG, W.K., AND WILHELM, G.	1953.	Publ. Health Rep. (Washington), 68, 985.
HARDY, M.H., BIGGERS, J.D., AND CLARINGBOLD, P.J.	1953.	Nature, 172, 1196.
HARRISON, R.G.	1907.	Proc. Soc. exp. Biol., 4, 140.
HOLT, S.J.	1959.	Exp. Cell. Res., 7, 1.
HUGGINS, C., JENSEN, E.V., AND CLEVELAND, A.S.	1954.	J. exp. Med., 100, 225.
JOST, A.	1939.	Ann. Physiol. Physiochim. Biol., 15, 1065.
JOST, A.	1943.	Ann. Endocr. (Paris), 4, 221.

KAHN, R.H. 1954. Nature, 174, 317.

KAHN, R.H. 1959. Ann. N.Y. Acad. Sci., 83, 347.

KAMAT, V.B. 1959. Personal communication.

KINGERY, L.B. 1921. J. Amer. med. Association, 76, 440.

KOCH, F.C., OTWAY, D., AND MUNSON, P.L. 1950. Endocrinology, 47, 73.

LANSING, A.I., AND OPDYKE, D.L. 1950. Anat. Rec., 107, 379.

LAZAROW, A. 1955. Rec. Prog. Hormone Res., New York, vol. 11, p. 254.

LEIGHTON, J. 1951. J. nat. Cancer Inst., 12, 545.

LENDRUM, A.C. 1947. J. Path. Bact. 59, 399.

LEUCHTENBERGER, C., AND LUND, H.Z. 1951. Exp. cell Res., 2, 150.

LITVAC, A. 1939. Arch. anat. Micr., 35, 55.

LUDFORD, R.J. 1930. In A System of Bacteriology in Relation to Medicine, London, vol. 7, p. 29.

LUSTIG, B., KATCHEN, B., AND REISS, F. 1958. J. invest. Derm., 30, 159.

LYELL, A., AND MILES, J.A.R. 1951. Brit. Med. J., 1, 912.

McCARTHY, L. 1931. In Histopathology of Skin Diseases, 1st Ed., London, p. 96.

McLAUGHLIN, A.I.G., AND EDINGTON, J.W. 1937. Lancet, 2, 685.

MANCINI, R.E. 1948. Anat. Rec., 101, 149.

MARSTON, H.R. 1946. In Fibrous Proteins - Proceedings of a Symposium held at the University of Leeds, May 1946, p. 207.

MARTIN, L. 1959. J. Endocr., 18, 334.

MARTIN, L., AND CLARINGBOLD, P.J. 1958. Nature, 181, 620.

MATOLTSY, A.G., AND SINESI, S.J. 1957. Anat. Rec., 128, 55.

MEDAWAR, P.B. 1947. Quart. J. micro. Sci., 88, 27.

MEDAWAR, P.B. 1948. Quart. J. micro. Sci., 89, 187.

MEDAWAR, P.B. 1953. Quart. J. micro. Sci., 94, 481.

MEIROWSKY, E., FREEMAN, L.W., AND WOODARD, J.S. 1954. J. invest. Derm., 22, 417.

MERCER, E.H. 1949. Biochem. biophys. Acta, 3, 161.

MIRAND, E.A., AND HOFFMAN, J.G. 1953. Exp. Med. Surg., 11, 63.

MISZURSKI, B. 1937. Arch. exp. Zellforsch. 20, 122.

MONTAGNA, W., EISEN, A.Z., RADEMACHER, A.H., AND CHASE, H.B. 1954. J. invest. Derm., 23, 23.

MOORE, G.E., BRACKNEY, E.L., AND BOCK, F.G. 1953. Proc. Soc. exp. Biol., 82, 643.

MOORE, T. 1957. In Vitamin A. Amsterdam, p. 550.

MORGAN, J.F., MORTON, H.J., AND PARKER, R.C. 1950. Proc. Soc. exp. Biol., 73, 1.

MORI, S. 1922a. J. Amer. med. Association, 79, 197.

MORI, S. 1922b. John Hopkins Hosp. Bull., 33, 357.

MORI, S. 1923. Amer. J. Hyg., 3, 99.

MUELLER, G.C. 1957. Cancer Res., 17, 490.

MÜTING, D., LANGHOF, H., AND WORTMANN, V. 1955. Ztschr. f. klin. Med., 152, 495.

NADEL, E.M., AND WODINSKY, I. 1955. J. Histochem. Cytochem., 3, 339.

NAIDU, V.R.	1935.	Proc. Mayo Clinic, 10, 356.
OAKLEY, C.L.	1938.	J. Path. Bact. 46, 109.
ODLAND, G.F.	1958.	J. biophys. biochem. Cytol., 4, 529.
PAUL, J.	1960.	In Cell and Tissue Culture, 2nd Ed., Edinburgh and London, p. 76.
PAULING, L., AND COREY, R.B.	1953.	Nature, 171, 59.
PEARSE, A.G.E.	1960.	In Histochemistry, 2nd Ed., London, pp. 117, 194, 265, 822, 825, 872.
PERCIVAL, G.H., DRENNAN, A.M., AND DODDS, T.C.	1947.	In Atlas of Histopathology of the Skin, Edinburgh, p. 420.
PERRY, V.P., SANFORD, K.K., EVANS, V.J., HYATT, G.W., AND EARLE, W.R.	1957.	J. Nat. Cancer Inst., 18, 709.
PINKUS, H.	1954.	In Rothman's Physiology and Biochemistry of Skin, Chicago, p. 590.
PORTER, K.	1954.	Anat. Rec., 118, 433.
PULLAR, P., AND COCHRANE, T.	1957.	Scot. med. J., 2, 189.
RAYNAUD, R.	1930.	C.R. Soc. Biol. (Paris), 104, 284.

REINERTSON, R.P., AND WHEATLEY, V.R.	1959.	J. invest. Derm., 32, 49.
ROBERTS, S., AND SZEGO, C.M.	1953.	Physiol. Rev., 33, 593.
ROBSON, J.M.	1936.	Proc. Soc. exp. Biol., 35, 49.
ROBSON, J.M.	1939.	J. Physiol., 96, 21.
ROBSON, J.M.	1950.	J. Endocr., 6, 449.
ROE, D.A.	1956.	J. invest. Derm., 27, 1.
ROE, D.A.	1956.	J. invest. Derm., 27, 319.
ROMANOFF, A.L.	1952.	Ann. N.Y. Acad. Sci., 55, 288.
ROSA, C.G., AND VELARDO, J.T.	1959.	Ann. N.Y. Acad. Sci., 75, 491.
ROTHMAN, S.	1954.	In Physiology and Biochemistry of the skin, 1st Ed., Chicago, pp. 344, 349, 354, 360, 366, 368.
ROUS, P., AND MURPHY, J.B.	1911.	J. Amer. med. Association, 56, 741.
RUDALL, K.M.	1946.	In Fibrous Proteins - a Symposium held at the University of Leeds, May, 1946, p. 15.

SALMON, U.J.	1939.	Proc. Soc. exp. Biol. (N.Y.), 41, 515.
SANFORD, K.K.	1958.	Cancer Res., 18, 747.
SANGIORGI, G.	1915.	Zentrabl. Bakt., 76, 257.
SCHILLER, J., DREYFUS, G., AND TYAN, E.A.	1953.	Ann. Endocr. (Paris), 14, 694.
SCOTHORNE, R.J., AND TOUGH, J.S.	1952.	Brit. J. plastic Surg., 5, 161.
SELBY, C.C.	1957.	J. invest. Derm., 29, 131.
SIEGEL, B.V., AND NOVY, F.G.	1955.	J. invest. Derm., 25, 265.
SMITH, C., AND PARKHURST, H.T.	1949.	Anat. Rec., 103, 649.
SPEAKMAN, J.B.	1936.	Nature, 138, 327.
SPEAKMAN, J.B., AND STOTT, E.	1938.	Nature, 141, 414.
SPEAKMAN, J.B., AND TOWNEND, F.	1937.	Nature, 139, 411.
STEELMAN, S.L., KELLY, T.L., NORGELLO, H., AND WEBER, G.F.	1956.	Proc. Soc. exp. Biol., 92, 392.
STRANGEWAYS, T.S.P., AND FELL, H.B.	1926.	Proc. Roy. Soc. Biol., 99, 340.

STRAUSS, M.J., BUNTING, H., AND MELNICK, J.L.	1950.	J. invest. Derm., 15, 433.
SWANBECK, G.	1959.	J. Ultrastructure Res., 3, 51.
TEMPLETON, H.J.	1935.	Arch. Derm. Syph. (Chicago), 32, 102.
TROWELL, O.A.	1954.	Exp. Cell Res., 6, 246.
TROWELL, O.A.	1959.	Exp. Cell Res., 16, 118.
TYAN, E.A., SCHILLER, J., PITOY, S., AND KONOPKO, B.	1953.	Ann. Endocr. (Paris), 14, 751.
UNNA, P.G., AND SCHUMACHER, J.	1925.	In Lebensvorgange in der Haut der Menschen und der Tiere. 1st Ed., Leipzig and Vienna, p. 26.
VAN SCOTT, E.J., AND FLESCH, P.	1954.	Arch. Derm. Syph. (Chicago), 70, 141.
VILLEE, C.A.	1959.	Ann. N.Y. Acad. Sci., 75, 524.
WADE, N.J., AND DOISY, E.A.	1935.	Proc. Soc. exp. Biol., 32, 707.
WARD, W.H., AND LUNDGREN, H.P.	1954.	In Advances in Protein Chemistry, Edited by M.L. Anson et al., New York, Vol. 9, pp. 243-297.

WASHBURN, W.W.	1954a.	J. invest. Derm., 23, 169.
WASHBURN, W.W.	1954b.	J. invest. Derm. 23, 97.
WEISS, P.	1953.	J. Embryol. exp. Morph., 1, 181.
WEISS, P., AND FERRIS, W.	1954.	Exp. Cell Res., 6, 546.
WEISS, P., AND JAMES, R.	1955.	Exp. Cell Res. Suppl., 3, 381.
WILE, U.J., AND KINGERY, L.B.	1919.	J. Amer. med. Association, 73, 970.
WILLIS, R.A.	1960.	In Pathology of Tumours. 3rd Ed., London, p. 268.
WILSON, H., BORRIS, J.J., AND BAHN, R.C.	1958.	Endocrinology, 62, 135.
WISLOCKI, G.B., FAWCETT, D.W., AND DEMPSEY, E.W.	1951.	Anat. Rec., 110, 359.
WOLBACH, S.B., AND HOWE, P.R.	1925.	J. exp. Med., 42, 753.
WOLBACH, S.B., AND HOWE, P.R.	1928.	Arch. Path., 5, 239.
WOODRUFF, A.M., AND GOODPASTURE, E.W.	1931.	Amer. J. Path., 7, 209.
ZUCKERMAN, S.	1940.	Biol. Rev., 15, 231.